
Carcinoma of the Cervix: Molecular Genetic Analysis

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ABSTRACT

Genetic alterations have now been identified in a variety of human cancers. This study has sought to examine some aspects of the molecular genetics of carcinoma of the cervix uteri.

The introduction presents a review of some important areas in the field of cervical carcinogenesis namely, epidemiology, aetiology, risk factors, and clinical aspects; genes and chromosomes in relation to carcinogenesis; and human papilloma viruses.

RFLP analysis was used to detect loss of heterozygosity in tumour/blood pairs, from patients with cervical carcinoma, to examine the role of commonly implicated tumour suppressor genes in cervical carcinogenesis. Allele losses were detected less frequently than has been reported in many other common solid tumours. This relatively low level of allele loss was supported by the infrequent genetic alteration identified when comparison was made between tumour/blood DNA fingerprints from cervical carcinoma patients and those with cancers of non-cervical origin.

No correlation was found between allele loss and HPV status when polymerase chain reaction (PCR) was used with DNA extracted from cervical carcinomas to detect HPV types commonly associated with genital lesions.

The mutational status of p53 was examined in a series of cervical carcinomas by a method employing PCR and denaturing gradient gel electrophoresis, and comparisons were made between the HPV and p53 mutational status of these tumours. Mutation in

p53 was detected relatively infrequently, and, contrary to recent reports, was not commonly associated with HPV negative status. Mutations were characterised by sequencing.

The use of p53 specific monoclonal antibodies in immunohistochemical analysis of normal, premalignant, and malignant cervical epithelium confirmed that p53 was seldom present in detectable quantities at any stage in the progression of this disease, and lent support to the finding of a low frequency of p53 mutations in this tumour type.

Y13 259, a monoclonal antibody to ras p21 oncoprotein, used to compare ras expression at various stages in cervical carcinogenesis, identified differences in expression in the glandular, but not the squamous, component of malignant and non-malignant cervical epithelium.

The correlation of various clinical and histopathological parameters, with flow cytometrically determined tumour DNA ploidy, revealed that patient age reached statistical significance when correlated with aneuploid tumour status.

It is suggested that cervical carcinogenesis is a multistep and multifactorial process, but that genetic mechanisms involved may differ from those that frequently operate in other common solid tumours. This is discussed in the context of existing knowledge, relevant comparisons are made with observations in other solid tumours, and proposed mechanisms of cervical carcinogenesis are reappraised.

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DECLARATION OF ORIGINALITY

I declare that the contents of this thesis have been composed by me, and that with the exceptions detailed below, the work conducted and described herein is entirely my own:

1. Cell lines developed from lymphocytes of patients with cervical carcinoma were established and maintained by Mrs Elizabeth Harvey, formerly of the MRC Human Genetics Unit in Edinburgh.
2. All tissue sections were cut by the technical staff of the Research Histology Laboratory of the Edinburgh University Pathology Department.
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5. Having been supplied with the PCR amplified p53 fragments, Dr Brian Cohen of the MRC Human Genetics Unit in Edinburgh performed denaturing gradient gel electrophoresis and sequencing.
6. Independent assessment of immunohistochemical staining in tissue sections was performed by Dr Alistair R.W. Williams.

I have not submitted this thesis in candidature for any other degree, diploma, or professional qualification.



R.M. Camille Busby-Earle

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PART I

INTRODUCTION

PART I INTRODUCTION

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1. A multistep mechanism for cervical carcinogenesis?
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CHAPTER 1

CERVICAL CARCINOMA

1. EPIDEMIOLOGY

1.1. *THE WORLD CANCER BURDEN*

On a world scale, cancer ranks third in importance as a cause of death. Indeed, a summary of mortality from 17 broad categories of causes (one such being neoplasms) in over 200 populations suggested that, in 1980, 4.2 million deaths in the world had been due to cancer. Overall, the number of cases is quite evenly distributed between developed (49.3%) and developing (50.7%) countries even though the ratio of populations is 1:3, implying that cancer is relatively more common in the developed than the developing world.

However, the absolute number of cases, and rankings of specific cancers are quite different across the globe. An estimate, for the year 1980, of the world cancer burden in terms of number of cases of cancer of 16 different types, in each of 24 geographical areas, revealed that there were approximately 6.35 million cases, of which 3.25 million were in men and 3.10 million in women (Parkin et al., 1988). One of the most striking features, however, was the frequency of two specifically female cancers - breast cancer and cervical cancer - which together account for one third of female cancers.

1.2. GLOBAL INCIDENCE OF CERVICAL CANCER

Cervical cancer is the second most common cancer among women worldwide. In 1980, there were an estimated 465,600 new cases, accounting for 15% of all cancers diagnosed in women. When all cancer sites for both sexes are combined, cervical cancer ranks fifth, accounting for 7.3% of all human cancers (Parkin et al, 1988).

However, the magnitude of the problem in different countries varies with the degree of development. In developing countries, this cancer ranks first even when both sexes are considered together; while in developed countries it ranks tenth. Indeed in some developing countries, cervical cancer is consistently the leading cancer in women, where it accounts for 24% of female cancers, and poses a major problem in areas such as sub-Saharan Africa and Latin America where it accounts for an even greater percentage.

1.3. GEOGRAPHICAL DISTRIBUTION OF CERVICAL CANCER INCIDENCE

A recent estimate of cervical cancer incidence revealed that 20% of cases occur in developed countries and 80% in developing countries (Parkin et al, 1988). The most recent data on invasive cancer incidence for selected areas of the world in which cancer registries are operating (Muir et al., 1987) which gave age standardised rate and the cumulative rate up to the age of 74 (the probability as a percentage of women developing invasive cervical cancer up to the age of 74) confirmed the high incidence rates for most developing countries.

Further, a study of the variations in cervical cancer incidence in the 24 United Nations geographical areas of the world showed that the regions of the world where the risk is highest are sub-Saharan Africa, Central and South America and South-East Asia (outside

Japan) where cancer of the cervix is the leading cancer, and constitutes 20-30% of all cancers in women.

The highest recorded incidence rates occur in South America, particularly in Recife in north-eastern Brazil (83.2 per 100,000). By contrast, cervical cancer accounts for only 4-6% of all female cancers in North America, Australasia and northern and western Europe. Low incidence rates are found in Middle Eastern populations, with a zone of relatively low risk (cervical cancer accounting for less than 10% of female neoplasms) appearing to extend from Pakistan to Egypt. The lowest rates occur in Israel (Jews - 4.0; non-Jews 3.0 per 100,000). Eastern Europe takes an intermediate position.

For large areas of the world, estimates are crude in that both the number of cases and the population size can be estimated only by indirect methods. Moreover, when extensive geographical areas are considered as a whole, differences that may exist between smaller units such as regions within a country, or between ethnic, religious or residential population groups may be overlooked.

Within countries, there are quite dramatic differences in incidence rates between regions and ethnic groups. The rates are generally 1.2-2.3 times higher in urban than in rural populations. An approximate two-fold difference has been detected between regions in Brazil, India, China, Switzerland and Canada (reviewed by Muñoz & Bosch, 1989).

1.4. INTER-POPULATION DIFFERENCES IN CERVICAL CANCER INCIDENCE

Among residents in Los Angeles, USA, the rate in Hispanic (Latino) women is almost three times greater than that in their Japanese counterparts. Likewise a range in incidence

rates is observed among the three ethnic groups in Singapore, and between the Maori and non-Maori populations of New Zealand.

Rates are also related to marital status and social class. They are higher in married women than in single, and higher in widowed and divorced women than in married women. Cervical carcinoma occurs extremely rarely in nuns (Gagnon, 1950). The incidence rates are about four times higher in the wives of unskilled than professional men. Further variations exist even within these groupings, for example the incidence of cervical carcinoma in the wives of clergymen is only 12% of the rate seen in other women of their age, while wives of seamen and fishermen have an incidence that is 160% that of their age-matched peers (Beral, 1974).

1.5. TIME TRENDS IN CERVICAL CANCER INCIDENCE

Data from cancer registries on the evolution of incidence rates for cervical cancer show a significantly decreasing trend since the early 1960s. The greatest declines are seen in countries where organised screening programmes have been introduced, among those age groups that are maximally screened (35-65) (Läara et al, 1987).

However, despite the existence of screening programmes, increases in incidence and mortality have been reported in young women in some countries since the late 1960s. In four cancer registries - German Democratic Republic, Norway, UK South Metropolitan and UK Birmingham - age specific incidence rates have shown a significant increase in young women, specifically in the age group 25-29 years. From other registries - USA: Alameda whites and New York State; Israel: all Jews; UK: South Western - non-significant increasing trends have been reported for women aged 20-24 and 25-29

(Muñoz & Bosch, 1989). Mortality rates have also shown an increase in young women in the UK, (Cook & Draper, 1984), Australia (Armstrong & Holman, 1981) and New Zealand (Green, 1979). However, no such increase has been observed in the USA (Chu & White, 1987), or France (Muñoz & Bosch, 1989), for women aged 35 and over.

Caution is necessary in the interpretation of time trend data from world cancer registries, as reporting artefacts may result from changes in screening coverage of the population, coding and registration practices of cervical cancer and carcinoma-in-situ, or hysterectomy rates. On the other hand, the increases observed in the UK have been evaluated taking into account the screening coverage of the population, and have occurred against a steadily decreasing trend observed in most Western countries since the late 1950s/early 1960s, and may suggest the introduction of some relatively new risk factor(s), which has its effect primarily on the younger generations, but whose impact is seen to varying extents across the world.

1.6. CERVICAL CANCER STATISTICS IN THE U.K.

In the UK cervical cancer ranks eighth in the list of most frequently occurring cancers, and accounts for 4% of female cancers. It accounts for 150-200 deaths per annum in Scotland and 1900-2000 in England and Wales. In 1985 there were 3970 cervical cancers registered in England and Wales with a further 11,755 registrations of cervical carcinoma-in-situ.

Evaluation of the screening programme in the UK has led to the conclusions that although little change has occurred in the national rates for the disease, screening has had a significant effect in reducing cervical cancer incidence, and therefore mortality (as

without screening, rates would have risen); but has only partially counteracted the trend for an increase in incidence and mortality among young women (Parkin et al., 1985).

2. AETIOLOGY AND RISK FACTORS

The aetiology of cervical carcinoma is still unknown, but there is reason to suspect that, like other neoplasms, both intrinsic and extrinsic factors play a role. There is a wealth of evidence for an association between cervical carcinoma and various behavioural risk factors, particularly indicators of sexual activity; and the roles of a few non-sexually related variables have also been examined (Davey Smith & Phillips, 1992). However, there are few data on the role of genetic susceptibility or predisposition, and unequivocal evidence for a causal relationship between cervical cancer and any of the risk factors so far identified is lacking.

2.1. *SEXUALLY RELATED FEMALE FACTORS*

Many of the associated risk factors relate to female sexual behaviour, and appear to be the inverse of those for cancer of the breast. In 1842, Rigoni-Stern reported that cervical carcinoma occurred essentially in married women; Gagnon in 1950 observed that squamous carcinoma was virtually non-existent in nuns; and an absence of reports on its occurrence in virgins has been noted.

More recent epidemiological studies have identified age at first intercourse, and independently the total number of sexual partners throughout life, as the two most clearly-defined and important risk factors for cervical carcinoma in western populations.

It is more common in women who have had multiple sexual partners and in those with a relatively early coitarche (Brinton et al., 1987; La Vecchia et al., 1986).

It has been suggested that frequency of intercourse may be directly related to an increased risk of cervical carcinoma. This may be intimately related to early coitarche and the number of sexual partners. Alternatively, its effect may be mediated through locally immunosuppressive effects of seminal fluid (see below), or the potential for genetic aberration in cells in which a high turnover rate has been stimulated by repeated trauma.

Intimately related to all these factors is multiparity. A consistent association between cervical carcinoma and multiparity has been identified independent of confounding sexual variables. The risk is increased by a factor of 1.4 for 4-5 births, but rapidly escalates to 5 fold for 14 or more, compared to 3 or fewer children. The association appears to be related to the total number of live births rather than pregnancies (Brinton et al., 1989).

The occurrence of cervical cancer in women claiming to have had only one sexual partner prompted a search for male related factors.

2.2. MALE PARTNER RELATED FACTORS

The roles of male sexual behaviour and male factors as determinants of risk for cervical cancer have been examined in various epidemiological studies.

An increased risk (7.8 for women with husbands with 15 or more partners extramaritally) has been described for women whose husbands reported multiple sexual partners (Buckley et al., 1981). In addition to the significantly increased risk associated with

promiscuity of the male partner, it has been shown that women married to men whose previous wives had had cervical cancer, have a two-fold increased risk of cervical cancer (Kessler, 1977). Wives of men with carcinoma of the penis show a 3-6 fold greater risk of cervical cancer than controls; and geographical clusters of high rates of both cervical and penile carcinoma have been described (Li et al., 1982).

It has been suggested that male circumcision and penile hygiene are correlated with a lower risk of cervical carcinoma amongst Muslim women (circumcised husbands) when compared to their Hindu counterparts in India, and the even lower incidence amongst Parsis (good penile hygiene) in the same region (Wahi et al., 1972).

Risks have also been associated with intrinsic male factors, particularly seminal fluid. Polyamines, eg spermine, present in high concentration in seminal fluid have been shown, in vitro, to interact with DNA of cervical cells, leading to changes in ploidy (Fletcher et al., 1991). This suggests that they may induce or promote dysplasia by chemical carcinogenesis. Prostaglandin E2 present in high concentration in seminal fluid is a potent immunosuppressant, and its locally immunosuppressive effects have been proposed as a factor in cervical carcinogenesis (Quayle et al., 1989; Kelly, 1991).

All these factors naturally confound the risks associated with female variables such as frequency of intercourse and number of partners. However the picture is even further complicated by the risks associated with contraceptive practices.

2.3. CONTRACEPTION AND SEXUAL HYGIENE

The relationship between various forms of contraception (oral, barrier, spermicidal) and

cervical neoplasia has been the subject of numerous epidemiological studies. Many of the studies on oral contraceptives ignored their correlation with other key risk factors such as number of sexual partners, age at coitarche and screening history, and yielded conflicting results.

The majority of studies which attempted to adjust for or control potentially confounding variables, suggested a significant increase in risk for cervical neoplasia in long term oral contraceptive (OC) users. On average, the increase in risk appears to be about two fold for OC use of more than 8-10 years, and a dose response relationship has been observed (Brinton et al., 1986). An increase in risk has been described not only for squamous carcinoma, but also for adenocarcinoma and adenosquamous carcinoma. It has also been postulated that OC use before the age of 20 could account for the increase in adenocarcinoma of the cervix seen in young women since the early 1970s in regions of the USA.

Rather than exerting a direct carcinogenic effect, it has been suggested that OCs may increase the risk by enhancing the effect of sexually transmitted carcinogenic agents. As a large body of evidence suggests the role of a sexually transmitted agent in the aetiology of cervical carcinoma, an effect of barrier contraceptives might be anticipated.

Such an effect has been described in studies which report a lower incidence of cervical neoplasia among diaphragm users than intrauterine device (IUD) or pill users (Wright et al., 1978). A protective effect of spermicidal contraceptives has also been demonstrated, and this is interesting in view of the virucidal effects of several spermicides. The strongest protective effect was observed for the use of contraceptive foams, creams or jellies, and

condoms.

An increased risk has been associated with both frequency and duration of vaginal douching, but how this relates to cervical trauma, chemical carcinogenesis or transport of sexually transmitted agents is as yet uncertain.

2.4. *INFECTIOUS AGENTS*

Evidence for an association between cervical cancer and sexual activity has been available for well over a century; and epidemiological data strongly implicate a venereally transmitted factor in its aetiology. Indeed, all the risk factors described thus far are fully compatible with venereal transmission of the essential aetiological factors and it has been suggested that many, if not all, of the sexual variables are surrogate measures of exposure to a putative sexually transmitted infectious agent causally related to cervical cancer.

Support for this theory also comes from the striking associations between the temporal, socio-economic and geographical distribution of mortality rates from cervical cancer, and the incidence rates of sexually transmitted diseases (Beral, 1974). The role of several viral agents including human papilloma virus (HPV), herpes simplex virus type 2 (HSV2) and cytomegalovirus (CMV) acting independently or synergistically has been examined.

The currently favoured hypothesis is that certain types of HPV play a key aetiological role. There is laboratory evidence to support this in that various methods have been successfully applied to detection of HPV in cases of preinvasive and invasive cervical carcinoma. However, while providing suggestive evidence, such series cannot serve as a basis for causal inferences. The hypothesis has been difficult to test epidemiologically, and

the results of studies which have been conducted remain controversial and inconclusive. HPV and its role in cervical carcinogenesis are discussed in greater detail in Chapter 3 of the Introduction.

Although recent studies tend to ignore the association, attention was previously focussed (for over 20 years) on the role of herpes simplex virus type 2 as an aetiological agent in cervical carcinoma. An independent oncogenic effect, a "hit and run" effect, and a synergistic effect with HPV have all been postulated, but results are inconclusive and its role as pilot, passenger or co-pilot remains to be clarified.

Studies on other infectious agents have included work on *Trichomonas vaginalis*, *Chlamydia trachomatis*, *Neisseria gonorrhoea*, *Gardnerella vaginalis*, *Ureaplasma urealyticum*, *Mycoplasma hominis*, *Candida albicans* and other yeasts. All have yielded conflicting results and conclusive evidence of a causal association between an infectious agent and cervical cancer is still lacking.

2.5. NON-SEXUALLY RELATED FACTORS

A search for a causal relationship between non-sexually related factors and cervical cancer has proved equally unrewarding. An association between cervical neoplasia and cigarette smoking, first proposed by Winkelstein (1977), has been reported in several studies. The level of risk in smokers when compared to non-smokers is generally of the order of 2, after adjustment for other confounding risk factors for the disease (Brinton et al., 1986). Chemical mutagens including cotinine and nicotine have been detected in the cervical mucus of smokers (Schiffman et al., 1987), making it biologically plausible that cigarette smoking could have a direct carcinogenic effect. The relative importance of its

effect compared to sexual variables remains controversial.

Few studies have explored the relationship between dietary factors and cervical neoplasia. An inverse relationship has been described between the risk of cervical carcinoma and each of the following - Vitamin C intake, Vitamin C plasma level, Vitamin A intake, retinol binding protein intake, β -carotene intake, and cellular levels of retinol binding protein. A diet rich in cruciferous vegetables and fat has been associated with an increased risk; and folic acid deficiency has been suspected as a factor associated with cervical cancer in women using oral contraceptives. The evidence for these appears suggestive but crude estimates of intake, and problems in control of putative relevant causal factors make cautious interpretation of these results mandatory (reviewed in Muñoz & Bosch, 1989).

Allied to diet, an association (similar to that seen for endometrial carcinoma) between excess weight and adenocarcinoma of the cervix has been claimed but may be the result of misclassification of uterine tumours when registered.

The potential role of local immunosuppression has already been mentioned with regard to PGE₂ in seminal fluid. (Quayle et al., 1989; Kelly, 1991). It is believed that general immunosuppression, whether primary or induced by therapy, conveys a high risk for viral infection, including HSV-2 and HPV and for the development of neoplasia. There is a 10-fold increased risk of cervical intraepithelial neoplasia (CIN) and cervical cancer in women with iatrogenic or systemic immunosuppression (Sillman et al., 1984). The HPV infection, which tends to be recalcitrant, is associated with neoplastic transformation, which runs a shorter course in progression to invasive cancer. The mechanism remains

uncertain. There is evidence to suggest an elevated risk (of the order of 4.7) for HPV infection and cervical carcinoma in renal transplant recipients (reviewed in Muñoz & Bosch, 1989).

Most reports on second or multiple neoplasms in patients have found associations between cervical cancer and other cancers (lung, larynx, buccal cavity, pharynx) thought to be associated with cigarette smoking. Their increased risk of cervical cancer may occur because of immunosuppression induced by their primary disease or by prescribed therapy. Alternatively, it may be due to the shared aetiological factors.

Pregnancy, a recognised period of transient immunosuppression, is associated with an increase in size of genital warts, and an increased prevalence of HPV (mostly HPV 16) compared to controls (Schneider et al., 1987). This may relate significantly to the increased risk associated with multiparity. It is possible that the increased risk of cervical cancer associated with immunosuppression is mediated by an increase in susceptibility to HPV or other viral infection or their effects, but this has not been confirmed.

2.6. PRE-INVASIVE DISEASE AND CERVICAL SCREENING

It is now accepted that squamous carcinoma of the cervix is preceded by cervical intraepithelial neoplasia (CIN). The presence of CIN in itself is therefore a risk factor for the development of invasive disease. Studies indicate that the behavioural risk factors for CIN are similar to those for invasive cancer.

There is a large body of evidence to suggest that cytological screening, if properly performed, can contribute markedly to the reduction in incidence and mortality rates of

cervical cancer (Parkin et al., 1985). It follows that for an individual woman, regular screening during adult life can, by identifying the lesion and facilitating treatment at the preinvasive stage, greatly reduce the risk of invasive cervical cancer.

3. CLINICAL ASPECTS

Cervical carcinoma is eminently preventable, and if diagnosed in the early stages, is easily treated with a good prospect of cure. Despite this, the morbidity and mortality of more advanced disease remains significant.

3.1. *HISTOLOGICAL CLASSIFICATION OF CERVICAL CARCINOMA*

Several types of neoplasm have been identified in the cervix, but carcinoma is by far the most common. There are 3 main histological types namely squamous carcinoma, adenocarcinoma, and adenosquamous carcinoma.

Squamous carcinoma is the most frequently occurring histological type, and for this reason, more is known about its natural history. Recently, an increase in the proportion of "glandular" (adeno- and adenosquamous) carcinoma of the cervix has been noted. This increase is believed to be the result of cytological screening, which, by detecting and facilitating treatment of preinvasive squamous lesions, has led to a decrease in the overall number of invasive squamous carcinomas. A similar decrease has not occurred for glandular carcinomas (reviewed by Jaworski, 1990).

A number of other histological types (eg. adenoid cystic, clear cell, glassy cell, papillary, small cell and carcinosarcoma) occur, but are relatively rare.

3.2. CIN AND THE NATURAL HISTORY OF CERVICAL CARCINOMA

The development of invasive squamous carcinoma of the cervix is believed to be preceded by identifiable preinvasive changes. These changes are known as cervical intraepithelial neoplasia or CIN, and are graded in increasing order of severity and propensity towards malignancy as CIN1, CIN2, and CIN3. It is believed that although spontaneous regression may occur at any of these stages, it becomes less likely as the severity of CIN increases. Furthermore, treatment at the preinvasive stage can prevent development of invasive cervical carcinoma and its sequelae.

Adenocarcinoma in-situ can also be identified histologically, but is less reliably detected clinically or cytologically.

3.3. CERVICAL CYTOLOGICAL SCREENING

Cervical cytological diagnosis depends on the detection of alterations in the morphology of cells, which can be appreciated only on good quality smears. Samples are taken with a spatula rotated through 360° on the ectocervix. There are several types of spatula available commercially. Special care is taken to include all of the transformation zone when sampling. The transformation zone, if visible, is the reddened area seen peripheral to the cervical os. It is the area in which the pubertal ectopy of columnar cells of the endocervical canal gradually undergo squamous metaplasia, on exposure to the acidic environment of the vagina. It is in this region of the cervix that cells seem most prone to undergo malignant transformation. Samples are smeared onto glass slides, fixed in 96%

alcohol, and stained. When cytological or clinical abnormalities are identified patients are referred for colposcopy.

3.4. COLPOSCOPY AND DIAGNOSIS OF CERVICAL CARCINOMA

At colposcopy, the cervix is inspected with binocular x16- magnification, prior to and after the application of 3% acetic acid or Lugol's iodine. Both these reagents, through colour changes of the mucosa, highlight abnormal areas of epithelium (acetowhite with acetic acid; non-staining pale Schiller's negative areas with iodine). In addition to fairly accurate demarcation of these abnormal areas, anomalous vascularisation in the form of punctation or mosaicism can also be detected. This permits sampling of areas with the most abnormal appearances and provides a biopsy for histological diagnosis.

Although most overt carcinomas of cervix can be diagnosed by an experienced clinician, accurate diagnosis and staging can only be achieved by a combination of biopsy, histology and clinical assessment.

3.5. STAGING AND SPREAD OF CERVICAL CARCINOMA

The staging of cervical carcinoma is intimately related to its pattern of spread which is well known. Spread is initially by local invasion, but is followed by progressive metastasis to lymph nodes. Untreated, metastasis to pelvic (external iliac, common iliac, and obturator) lymph nodes is usually followed by involvement of para-aortic, thoracic, and eventually supraclavicular nodes. Despite local control, recurrence and death from cervical carcinoma may occur from failure to control regional and distal tumour spread.

The most widely used clinical staging follows the guidelines of the International

Federation of Gynaecology and Obstetrics (FIGO) which are outlined below :

Stage 0	Carcinoma in situ
Stage I	Carcinoma confined to the cervix
Stage II	Carcinoma extends beyond the cervix, but not to the pelvic side wall or lower third of the vagina
Stage III	Carcinoma extends to the pelvic side wall, and/or the lower third of the vagina, and/or causes hydronephrosis or non-functioning kidney
Stage IV	Carcinoma extends beyond the true pelvis or clinically involves the bladder or rectal mucosa

Within these broad categories several subcategories exist :

IA	Preclinical carcinoma diagnosed by microscopy
IA1	Minimal microscopically evident stromal invasion
IA2	Measurable (depth $\leq 5\text{mm}$; horizontal spread $\leq 7\text{mm}$) microscopically detected lesions
IB	Lesions larger than IA2 whether seen clinically or not
IIA	No parametrial involvement
IIB	Parametrial involvement
IIIA	Involves lower third of vagina but not pelvic wall
IIIB	Involves pelvic wall and/or compromises renal function

3.6. PRESENTATION OF CERVICAL CARCINOMA

In countries with organised screening programmes, and where population awareness is high, disease is usually detected in the early invasive or preinvasive stages. For most of the developing world, the converse is unfortunately true.

Patients usually present in one of two ways. They are either asymptomatic, having had abnormalities detected on routine cervical screening or pelvic examination; or alternatively, symptoms of the invasive cancer in the form of abnormal vaginal (intermenstrual, postcoital or postmenopausal) bleeding or offensive discharge provide the alert. In late stages of the disease, patients may present with unusual or non-specific symptoms (fistulae, pyrexia, urinary retention, haematuria, renal failure) indicative of involvement of other organ systems by tumour.

3.7. TREATMENT OF PREINVASIVE AND INVASIVE DISEASE

Treatment options vary with clinical and histological diagnosis, staging and clinical presentation.

Preinvasive disease may be treated by either local ablation (laser, cryotherapy, cold coagulation) or by excision. Excision may be local (cone biopsy or diathermy loop excision of the transformation zone); or simple hysterectomy may be performed, if other gynaecological symptoms indicate and reproductive function is no longer required.

Treatment options for invasive cervical cancer are essentially surgical and radiotherapeutic, although trials are currently underway to assess the merit of adjuvant chemotherapy in palliation of advanced disease. Radiotherapy is the undisputed primary treatment of choice for disease more advanced than Stage II. Either surgery or radiotherapy may be used to treat Stage I and early Stage II disease. The choice of therapy is often influenced by the tumour size, the skill of the surgeon, the experience of the radiotherapist, the availability of hospital facilities and equipment, and patient factors

such as age, performance status, and individual preference.

In practice, a substantial proportion of Stage IB tumours are treated by radical Wertheim's hysterectomy, involving parametrial and deep pelvic lymph node dissection, with removal of a large vaginal cuff of tissue. Surgical treatment in the form of pelvic exenteration may be required in the management of more advanced or recurrent tumours. In some cases, adjuvant radiotherapy is used to treat residual disease; and, depending on the size, location, and nature of recurrences, either surgery or radiotherapy may be employed.

3.8. *SURVIVAL DATA*

The five year survival of patients with cervical carcinoma after treatment is shown below (Hernandez & Rosenshein, 1989), but these figures are further influenced by the nodal status at the time of treatment.

STAGE	5 YR. SURVIVAL(%)
I	92
IIA	84
IIB	67
IIIA	45
IIIB	36
IV	14

CHAPTER 2

GENES, CHROMOSOMES AND CANCER

1. CANCER AS A GENETIC DISEASE

1.1. *BACKGROUND*

It is now widely accepted that cancer is ultimately a disease of the genome (Seemayer & Cavenee, 1989; Weinberg, 1989; Benz, 1990; Stoler, 1991). A number of early observations excited interest in the role of cellular genetic material in carcinogenesis. Firstly it was observed that all carcinogenic agents - chemicals, radiation, viruses - are potentially mutagenic and cause damage to cellular DNA. Secondly it was noted that patients with the rare inherited disorders of DNA stability such as xeroderma pigmentosum, ataxia telangiectasia, Fanconi's anaemia and Bloom's syndrome, show an increased susceptibility to malignant tumour development (reviewed in Steel, 1989).

In addition, cytogenetic analysis of tissue from patients with malignancy often revealed structural and numerical alterations to the normal chromosomal complement. In keeping with this genetic association, the identification of familial cancers such as retinoblastoma suggested the existence of a heritable genetic defect which conferred increased susceptibility to these tumours.

1.2. CYTOGENETIC EVIDENCE

With improvements in cytogenetic techniques, several chromosomal abnormalities have come to be consistently associated with certain cancers. Among them are the reciprocal translocation between chromosomes 9 and 22 [t(9;22)(q34;q11)] with the generation of the Philadelphia chromosome (#22 with short arm deletion) in patients with chronic myeloid leukaemia (Rowley, 1973) and the reciprocal translocation between chromosomes 8 and 14 [t(8;14) (q24;q32)] (Manolov & Manolova, 1972) or chromosomes 8 and 2p13 or 22q11 in Burkitt's lymphoma (Croce et al, 1984). In fact it has been noted that the majority of cancers have a demonstrable cytogenetic defect (Yunis, 1983).

However, cytogenetic analysis is a relatively crude method of detection of genetic alterations. By revealing gross structural or numerical chromosomal defects it serves as a guide to chromosomes of potential interest, but is unable to detect more subtle genetic alteration which may occur at the submicroscopic level.

1.3. MOLECULAR EVIDENCE

The recent development of elegant and sophisticated molecular biological techniques for DNA analysis has enhanced our understanding of genetic alterations in carcinogenesis. First, it has permitted more detailed analysis of cytogenetically detected aberrations, eg the Burkitt's lymphoma translocation has been shown to bring the *c-myc* oncogene (important in the control of DNA replication and transcription) into close association with, and hence under the influence of immunoglobulin heavy and light chain gene promoter/enhancer elements from chromosomes 2, 14 or 22, important in B cell differentiation (Croce et al, 1984).

Secondly, it has led to the detection of submicroscopic changes in DNA undetectable by cytogenetic analysis. A host of genetic changes have been identified as frequent and often type-specific events in human cancers. Further, the worldwide quest for clues to the mechanisms of carcinogenesis has generated a wealth of information on the type, frequency, clustering and potential effects of specific genetic changes in diverse cancers.

1.4. *PROPOSED MECHANISMS*

Some general conclusions have evolved. Carcinogenesis is a multifactorial and multistep process involving several alterations in specific genes, in particular activation of oncogenes and inactivation of tumour suppressor genes (TSG) (Bodmer, 1988; Solomon et al., 1991; Steel, 1989; Wynford-Thomas, 1991). These changes are manifest as loss of control of growth, replication and differentiation with the acquisition of invasive and metastatic potential - features which are characteristic of transformed and malignant cells. Finally, tumorigenesis can result from such genetic changes occurring in a single cell which then has the potential for unlimited clonal expansion and development of a malignant lesion.

2. ONCOGENES AND CANCER

The search for genes associated with carcinogenesis has led to the identification of a group of genes known collectively as oncogenes which by their presence contribute or have the potential to contribute to tumour development.

2.1. VIRAL ONCOGENES

The initial clues to the identification of oncogenes came from observations in certain oncornaviruses known as retroviruses (small RNA viruses associated with tumour development in animals). When compared to other RNA viruses, these oncogenic RNA viruses have an additional sequence - the onc sequence (*v-onc*) in their genome which has been shown by mutation and deletion to confer oncogenicity. It is further known that these viruses require to make a DNA copy of their genome which becomes integrated into the host DNA for completion of the viral life cycle. It was therefore no surprise when *v-onc* sequences were identified in the host cell DNA of retrovirally induced animal tumours. It was further discovered that the transfection of DNA copies of these *v-onc* sequences resulted in transformation of cultured cells in vitro, and that these cells in turn could effect tumour development when inoculated into susceptible animals.

Over 30 such viral oncogenes (*v-onc* sequences) have now been described, and each has been given an abbreviated 3 letter name based on the type of tumour and the animal in which the virus was first described. Thus *v-src* refers to the viral oncogene found in the sarcoma virus first described by Rous (Rous, 1911) in chickens. There followed many others including *v-ras* (rat sarcoma virus), *v-myc* (avian myelocytomatosis virus), *v-abl* (Abelson mouse leukaemia virus), and so on. Where an oncogene has been identified in different strains of the same virus, its 3 letter name is qualified by the name (usually that of its discoverer) of the viral strain, so that *v-Ha-ras* describes the *ras* gene from the strain of rat sarcoma virus described by Harvey, while *v-Ki-ras* refers to its counterpart from the Kirsten strain.

Certain large DNA viruses also have oncogenic associations with human cancers (Epstein

Barr virus and Burkitt's lymphoma; hepatitis B virus and hepatocellular carcinoma; HPV and cervical carcinoma), and are believed to possess oncogenic sequences.

The relevance of viral oncogenes to human carcinogenesis came with the identification of normal human cellular counterparts of *v-onc* sequences (reviewed in Steel, 1989).

2.2. CELLULAR ONCOGENES

Sequences almost identical to *v-onc* sequences have been identified in normal (and tumour derived) human genomic DNA. In this context they have been termed *c-onc* (cellular oncogenes) to distinguish them from their viral counterparts. Transfection of tumour-derived cellular sequences, like those derived from their viral counterparts, has been used to successfully transform cell lines, which are then capable of producing tumours in mice *in vivo*.

The first *c-onc* sequence to be discovered was that of *c-Ki-ras*, but since then several oncogenic sequences have been identified in normal and tumour DNA. Many including *c-myc*, *c-abl*, *c-ras* and *c-src* have been identified because of their homology to viral oncogenes. Most of these sequences are highly conserved evolutionarily and it is believed that retroviral sequences are derived from cellular sequences picked up by the virus during passage through an infected cell, perhaps because of some conferred survival advantage. However not all *c-oncs* have been identified in this way. Some (*ras*, *myc*) have been identified by their ability to transform rodent cells by DNA transfection; others (*N-myc*, *erb-B2*) by their amplification (multiple gene copies) or overexpression (excessive transcription) in human tumours; others (*c-abl*, *bcl-1*, *c-myc*) by their location at tumour specific chromosomal breakpoints; and some (*myc*, *abl*, *ras*) have been

identified by several of these routes.

Clues to the putative role of oncogenes in human carcinogenesis have been provided by the results of several experimental studies including work on transgenic mice (Adams & Cory, 1991). Indeed, results of studies examining the products encoded by these genes, their function, and sites of action have gone some way in unravelling the role of some oncogenes in carcinogenesis.

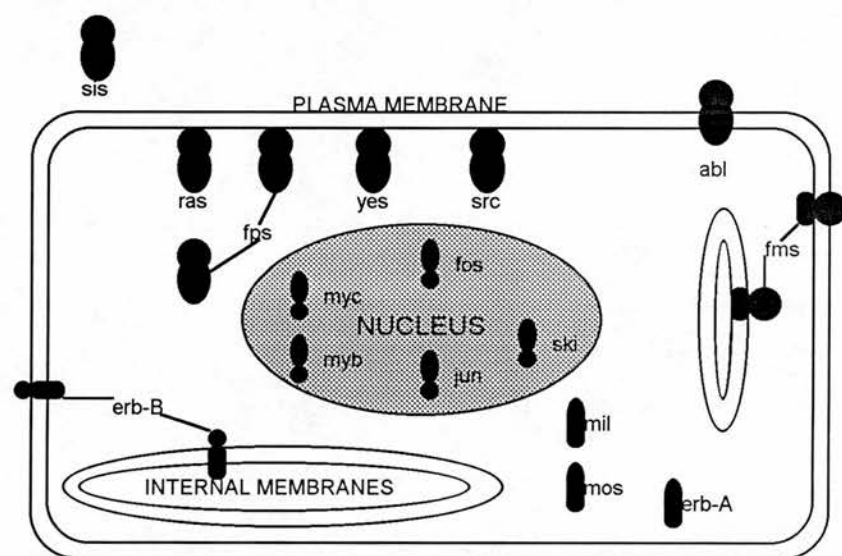
2.3. CELLULAR FUNCTIONS OF ONCOGENES

Various functions have been assigned to oncogenes, but they all appear to play some role, directly or indirectly, in cell cycle control, and hence in cell proliferation and differentiation. Products encoded by oncogenes include growth factors (eg. *sis* and platelet derived growth factor) and their receptors (eg. *erb* B-2 and epidermal growth factor receptor); cellular signal transducers including protein kinase and phosphorylation dependant GTP binding proteins (eg. *ras*); tyrosine kinases (eg. *abl*, *src*, *fes*, *raf*, *mos*); and DNA binding proteins (eg. *myc*, *jun*) which may function in DNA transcription and replication (reviewed in Steel, 1989; Wynford-Thomas, 1991).

Specific sites of action of oncogene products have been identified throughout the cell. Some growth factors (eg. *sis*) act on extracellular membrane receptors. Many receptors and signal transducers are membrane bound. Some oncogene products function as transmembrane proteins of the plasma membrane (eg. *abl*); while others (eg. *erb* B2, *fms*) have been detected as transmembrane proteins in both the external and internal plasma membranes of the cell. Certain signal transducers or transmitters (eg. *ras*, *yes*, *src*) seem to function solely on the cytoplasmic side of the plasma membrane; others (eg. *mil*, *mos*,

erb A) are entirely cytoplasmic in action; while others (eg. *fps*) act at both sites. The actions of other oncogenes (eg. *myc*, *myb*, *fos*, *jun*, *ski*) appear to be confined to the cell nucleus (see Fig. 1).

Fig. 1. Sites of action of oncogene products



2.5 MECHANISMS OF ACTIVATION

Several mechanisms have been proposed whereby in tumorigenesis these oncogenes become activated in such a way that their normal cellular function is qualitatively or quantitatively altered. These activating mechanisms include structural changes, amplification and dysregulation.

Structural changes in both the host and viral oncogenes are believed to be important. In viral acquisition of cellular oncogenes, sequences have undergone structural changes that

account for *v-onc/c-onc* differences. Cells infected by retroviruses, therefore have not only an increased copy number of an oncogene, but often an integrated copy of a structurally altered *v-onc* sequence.

Structural changes in the form of single point mutations can alter the structure and function of the gene product (eg. some *ras* mutations); more major structural changes can result in the production of a truncated gene product that has altered activity (eg. *v-src* vs. *c-src*; *v-fms* vs. *c-fms*); and gene splicing can result in the creation of new sequences, the fusion protein products of which may have altered function when compared to the *c-onc* (eg. the *abl/bcr* transcript).

Gene amplification has also been proposed as a mechanism of oncogene activation. While structural changes have been shown to alter the function of the gene product, it has been shown that a change in gene dosage, such as gene amplification, which can result in increased quantities of a structurally normal oncogene product may also disrupt normal cellular function (eg. *erb B2* in breast cancer).

Dysregulation, involving alterations to the regulatory controls (promoters/enhancers) of oncogene expression can also result in a quantitative change in a structurally normal gene product without necessarily requiring gene amplification (multiple gene copies). An example of such oncogene overexpression is seen in the dysregulation of the *c-myc* gene mediated by a chromosome 8 to 2, 14 or 22 translocation which brings the normal cellular oncogene under the transcriptional control of immunoglobulin gene promoter/enhancer elements, and which results in overexpression of a normal *myc* product in Burkitt's lymphoma (Croce et al, 1984). Dysregulation of structurally altered

genes may also occur. For example, integrated altered viral sequences may come under the control of promoting host sequences and potentially result in a gene product that is both qualitatively and quantitatively altered.

Oncogene activation has now been described in several human cancers including *ras* in colon cancers, *erb B2* in breast cancers, and *myc* in lymphomas. However, more recently it has been found that in malignant tumours oncogene activation often co-exists with alterations in another group of genes known as "anti- oncogenes" or tumour suppressor genes. It is now believed that the effects of alterations in both groups of genes may be required in the multi-step process of carcinogenesis (Steel, 1989; Wynford-Thomas, 1991).

3. TUMOUR SUPPRESSOR GENES (TSGs) AND CANCER

Loss or inactivation of certain genes has been identified as characteristic of many malignancies, and has led to the identification of a group of genes now known as tumour suppressor genes (TSGs), which appear to contribute to the suppression of tumorigenesis (Green & Wyke, 1985; Friend et al., 1988; Goudie, 1988; Green, 1992).

3.1. BACKGROUND

Although most of the recent data on TSGs has come from studies on solid tumours, the initial clues to their existence came from cell fusion experiments. Somatic cell hybrids, derived from the fusion of malignant cells with normal parental cells, were found to be transformed but non-tumorigenic. Further, the evolution, in time, of tumorigenic

segregants from these hybrids was associated with and occurred as a consequence of loss of specific normal chromosomes (Harris, 1988).

The second line of evidence came from Knudson's observations and statistical analysis of the genetics of retinoblastoma development (Knudson, 1971). Retinoblastoma is a rare childhood cancer which exists in two forms - a familial form which usually displays autosomal dominant inheritance, and a sporadic form. Early age of onset, multifocal and bilateral lesions, and an increased incidence of second neoplasms, especially osteosarcomas, but also including breast cancers, bladder and lung cancer, and melanomas, are characteristic of the familial form. The sporadic form tends to occur at a relatively later age, and solitary lesions are typical.

In his analysis, Knudson proposed a "two-hit" mechanism of carcinogenesis (Knudson, 1971). He postulated that in familial retinoblastoma the first "hit" took the form of a germ line mutation which affected all cells, while the second "hit" occurred as a somatic event targetted at the same locus in the remaining normal allele of that gene in a retinoblast. This led ultimately to tumour development in that cell. He further argued that in the sporadic form both "hits" occurred as somatic events in the same retinoblast. The first did not alter the phenotype of the cell as one normal gene copy would be sufficient to suppress tumorigenesis. However, the second event, which affected the remaining and corresponding normal allele of the gene, in that retinoblast would trigger tumour development.

The relevance of Knudson's hypothesis was not immediately realised, but its proposals have since been supported by cytogenetic and molecular genetic analyses, which have

identified regions of certain chromosomes that are consistently lost, mutated or deleted in specific cancers, including retinoblastoma.

3.2. THE RETINOBLASTOMA TUMOUR SUPPRESSOR GENE

Comparison of constitutional and tumour genotypes in retinoblastoma patients has consistently identified chromosomal events (allele loss, deletion or mutation), many not cytogenetically obvious, in the 13q.14 region, and has led to the isolation of the Rb gene thought to be associated with retinoblastoma (Friend et al., 1986). Similar studies on other familial and many sporadic cancers have demonstrated that this and other putative tumour suppressor genes are also consistently lost or mutated (Heim & Mitelman, 1989).

The retinoblastoma (Rb gene) model has been used to deduce possible mechanisms by which TSGs may be involved in other cancers. It would seem that in familial retinoblastoma, the inherited defect inactivates one copy of the Rb gene, rendering the individual functionally hemizygous at the Rb locus in all cells; and that tumorigenesis is triggered by chromosomal loss or deletion of the remaining normal Rb gene in a retinoblast. In sporadic disease, a retinoblast with normal Rb genotype becomes hemizygous at this locus after the first somatic event, but tumour development occurs only when Rb function is lost in this cell by targetting of the second event at the remaining normal allele. In both cases, tumorigenesis is triggered by the homozygous inactivation of the Rb gene in a retinoblast.

It has therefore been deduced that TSG behaviour is recessive (both copies need to be inactivated) with regard to tumorigenesis, but dominant (only one copy being required) in terms of tumour suppression (Ponder, 1988; Mitchell, 1991; Weinberg, 1991). Gene

inactivation occurs by submicroscopic mutational mechanisms or by loss of the gene, its region, or its chromosome. Homozygous inactivation often occurs by a combination of both mechanisms, so that a localised lesion (point mutation or small deletion) is followed by a more extensive loss of sequence from the homozygous allele (Wynford Thomas, 1991). This facilitates detection in that the presence of the mutationally inactivated allele may still be detectable by molecular genetic means when its counterpart has been deleted.

In fact, this is one of the principles underlying the interpretation of one of the most commonly used techniques in the study of TSGs - allele loss analysis by the detection of loss of heterozygosity.

3.3. IDENTIFICATION OF TUMOUR SUPPRESSOR GENES

Several techniques have now been used in the identification of putative TSGs. Before the advent of molecular genetic techniques, identification of sites of potential TSGs was based on detection of consistent chromosomal deletions by a combination of karyotypic analysis and pedigree analysis of inherited tumours, eg. retinoblastoma and Wilm's tumour. However advances in detection of deletional events came with the development of hybridisation techniques and the discovery of DNA polymorphisms.

Polymorphism refers to the variation in length (on size fractionation) of homologous DNA (restriction fragment) sequences generated on enzymatic DNA digestion. The variation arises as a result of differences in restriction sites on the lengths of intervening non-coding sequences in maternally and paternally derived alleles. Their recognition has led to the development of highly polymorphic DNA probes which detect sequences that show heterozygosity (distinguishable maternal and paternal alleles) in most individuals.

This has made restriction fragment length polymorphism (RFLP) analysis, the cornerstone of TSG analysis and allowed closer scrutiny of the genome (Landegren et al., 1988; Mitchell, 1991).

In RFLP analysis, labelled polymorphic probes are used as markers to identify individuals constitutionally heterozygous for specific loci. By comparing constitutional DNA with tumour DNA from the same individual, allele loss can be detected as loss of heterozygosity (LOH). The steps leading to homozygous inactivation of a TSG allele usually involve the flanking chromosomal sequences as well (Weinberg, 1991). Accordingly, mapping of the region involved can be achieved by comparing the pattern of LOH at multiple loci in tumours from a series of patients. In this way, new potential TSGs can be located, and further characterised.

The repeated observation of LOH of a specific chromosomal marker(s) in cells from a specific tumour type suggests the presence of a closely mapping TSG, the loss of which is involved in tumour pathogenesis (Weinberg, 1991). Indeed, such mapping exercises have been powerful tools in delineating parts of the genome and have led to the cloning of four tumour suppressor genes - Rb1, p53, DCC and APC (Oren, 1985; Friend et al., 1986; Bodmer et al., 1987; Fearon et al., 1990).

The suppression of tumorigenicity of transformed cells and tumour cell lines by microcell transfer of specific chromosomes or parts thereof has also been used as a clue to the location of putative TSGs (Saxon et al., 1986). Once cloned, they have been used to develop transgenic mice (the progeny of a pseudo-pregnant surrogate mouse implanted with a fertilised mouse embryo into which a mutant gene of interest has been transfected)

in an attempt to examine the effects of mutation in TSGs.

Using these techniques, several putative tumour suppressor genes have been identified (Ponder, 1988; Sager, 1989; Weinberg, 1991). They include the prototype retinoblastoma TSG, Rb1 located at 13q.14; two possible Wilm's tumour WT genes sited at 11p.13 and 11p.15; the p53 gene at 17p.13; the neurofibromatosis NF1 gene on 17q; a gene PTGP, or genes in the region 3p.21 commonly deleted in small cell lung carcinoma and renal carcinoma; the DCC gene deleted in colorectal cancer located at 18q.21, and the APC gene of familial adenomatous polyposis coli, found in the 5q.21 region (reviewed in Weinberg, 1991; Solomon et al., 1991).

3.4. *FUNCTION OF TSG PRODUCTS*

Recent experiments have shed light on the products of some oncosuppressor genes, but as yet their precise functions in cell cycle control, growth, differentiation and proliferation remain unclear. Indeed it has been difficult to reconcile their biochemical functions with their biological effect of tumour suppression.

The Rb1 gene encodes a ubiquitously expressed 105kDa DNA-binding nuclear phosphoprotein, pRb, that appears to be involved in transcriptional regulation and cell cycle control. pRb is capable of forming complexes with the oncoproteins of some oncogenic DNA viruses including SV40 large T antigen, and E7 of human papilloma virus (HPV) (Dyson et al., 1989). One possibility is that this binding cripples pRb function, and may account for the oncogenicity of these viruses.

WT-1 is a highly tissue specific and developmentally regulated gene encoding a 345

amino acid protein with the hallmarks of a transcription factor. It has four zinc finger proteins which indicate a sequence specific DNA binding protein (reviewed in Weinberg, 1991).

It has been postulated that the p53 gene, initially described as an oncogene, is expressed in appreciable quantities only in cells with DNA damage. It encodes a 53 kDa nuclear protein which acts as a transcription factor and whose regulation of the cell cycle is mediated by G1 arrest in cells with DNA damage, switching off replication to allow DNA repair or apoptosis (Lane, 1992).

The NF-1 gene product bears some homology to GTPase activating proteins, and a role in signal transduction via regulation of the p21 protein of the *ras* proto-oncogene is believed relevant to cell differentiation and tumorigenesis (Weinberg, 1991). A potential 3p.21 candidate gene, PTGP encodes a receptor protein with tyrosine phosphatase activity (reviewed in Solomon et al., 1991).

The DCC gene encodes a 190 kDa transmembrane phosphoprotein with the attributes of a cell surface receptor (Fearon et al, 1990). It bears particular homology to neural cell adhesion molecules (N-CAMs). Its role as a signal transducing receptor, inactivation of which may lead to a loss of cell adhesion and invasive and metastatic potential has been proposed (Fearon et al, 1990).

3.5. *LOSS OF TSGs IN HUMAN CANCERS*

From RFLP analysis studies, there is now evidence for allele loss in the vicinity of TSGs in many human malignancies, and the association between allele losses on specific

chromosomes in particular cancers has proved highly significant. Loss of heterozygosity (LOH) at putative TSG sites has now been demonstrated in diverse familial and sporadic cancers. While allele losses at some TSGs have been identified in several types of tumour, others have been relatively tumour-type specific, and LOH at multiple TSG sites has been found in some cancers.

Losses have been observed in cancers of breast (17p, 17q) (Mackay et al., 1988a; Sato et al., 1991); colon (5q, 17p, 18q) (Vogelstein et al., 1988, Fearon et al., 1990; Ashton-Rickardt et al., 1991; Purdie et al., 1991); ovary (17p, 17q) (Eccles et al., 1990; Russell et al., 1990), lung (3p, 5q) (Naylor et al., 1987; Mori et al., 1989; Ashton-Rickardt et al., 1991), kidney (3p) (Zbar et al., 1987), bladder (9q, 11p, 17p) (Tsai et al., 1990), brain (17p) (Fulst et al., 1989) and bone (13q, 17p) (Toguchida et al., 1988; 1989).

Of these, it is chromosome 17p which has probably received the most attention and publicity, and, to date, mutation in p53 (17p.13) is the most commonly detected molecular defect in human cancers (Wynford-Thomas, 1991).

4. p53 GENE AND CANCER

4.1. BACKGROUND

The p53 gene is a tumour suppressor gene (TSG) which has been located on the short arm of chromosome 17 at 17p.13.1, and which has been the focus of much research over the past decade. Three aspects of the p53 scenario have justified this focus. Firstly, p53 appears to be involved through mutation or deletion in 40-50% of diverse human cancers.

Secondly, it seldom seems to adhere to the principles now used to define other TSGs; and thirdly, its precise role in tumorigenesis seems complex, and has proven difficult to clarify.

The gene has been isolated from man, monkey, rat, frog, chicken and bony fish (Lane & Benchimol, 1990). It comprises 11 exons separated by 10 introns (Montenarh, 1992), and phylogenetic studies which have demonstrated its conservation in vertebrates have also identified 5 regions which have been highly conserved throughout evolution. These highly conserved domains (HCDs) I-V correspond to exons 2 (I), 4 and 5 (II), 6 (III), 7 (IV) and 8 (V) (Soussi et al., 1990). The gene encodes a highly conserved 393 amino acid, 53kD nuclear phosphoprotein.

Its initial discovery in 1979 (Lane & Crawford, 1979), in association with the SV40 large T antigen in virus transformed cells, and later as an overexpressed antigen in chemically transformed sarcoma cells, led to its initial and possibly erroneous classification as an oncogene. It was subsequently found that wild-type (WT) p53 cDNA suppressed growth and inhibited transformation (Baker et al., 1990a); while p53 cDNAs that cooperated with cotransfected ras oncogene to transform embryo fibroblasts was found to be mutant (Eliyahu et al., 1988). A distinction has therefore since been made between WT (growth suppressive) p53 and mutated (growth stimulatory) p53 in terms of their characteristics, functions, and roles in tumorigenesis.

4.2. *WILD TYPE p53*

WT-p53 is a nuclear phosphoprotein involved in transcriptional regulation. It has a transcriptional domain and displays sequence-specific DNA binding. It assembles into

homotetramers and higher order homo-oligomeric structures within the cell, and has a short half-life of only 20-30 mins (Lane & Benchimol, 1990). Its short half-life dictates that in normal cells p53 is found only in small quantities which are undetectable by conventional immunocytochemical methods. It interacts with the heat shock protein, Hsc 70 but these interactions are transitory, and do not lead to accumulation of the protein.

Like pRb, WT-p53 binds viral oncoproteins like SV40 large T antigen, adenovirus E1B and human papilloma virus HPV E6. Complexes between p53 and large T antigen or E1B sequester p53 in inactive complexes, prevent it from reaching its normal site of action in the nucleus and increase steady state cellular WT- p53 concentrations. The interaction between p53 and HPV E6, on the other hand, results in its rapid destruction by a ubiquitin- dependant cellular pathway, so that accumulation of the protein does not occur (Scheffner et al., 1990, 1991; Montenarh, 1992).

4.3. *MUTATED p53*

Mutated p53 has different characteristics. Like WT-p53 it can oligomerise with WT-p53, altering its oligomeric conformation, and leading to stable multi-unit complexes which accumulate in the cell. It also complexes to Hsc 70 heat shock protein leading to the accumulation of unproductive, often cytoplasmic complexes. Complexes involving all three moieties occur and result in sequestration of active p53 that is then unable to reach its normal nuclear site for growth regulation, and the accumulation of stable inactive p53 complexes with greatly increased half- lives (Montenarh, 1992). Mutated p53, by its accumulation in the cell, with a long half-life, is therefore detectable by immunohistochemical methods, and several monoclonal antibodies have now been developed (Banks et al., 1986; Gannon et al., 1990; Midgeley et al., 1992; Vojtesek et al.,

1992).

Mutated p53, perhaps because of self-oligomerisation or interactions resulting in WT-p53 sequestration, has been shown to act as a dominantly transforming oncogene (Lane & Benchimol, 1990) in that, when both mutated and WT-p53 are present in the same cell, the effect of the mutant species overrides that of the WT. It has further been described as having a dominant negative effect in that, through mutation, it not only loses its suppressor function (by heterodimer formation), but also inhibits the function of any normal p53 present in the cell.

Over 300 mutations in the p53 gene have now been described in human cancers (Caron de Fromental & Soussi, 1992). 66% of these p53 mutations in diverse human cancers have occurred in the 4 "hot-spot regions" (HSRs) A-D which coincide with HCDs II-V, previously described by Nigro et al. (1989), while these 4 HSRs and a fifth HSR (designated HSR A') accounted for 73% of the mutations detected. HSR A' is not in a classical HCD, but is located in a region conserved during mammalian evolution (Caron de Fromental & Soussi, 1992).

4.4 EVIDENCE OF p53 INVOLVEMENT IN TUMORIGENESIS

There is a wealth of evidence implicating p53 in tumorigenesis, and loss of function of p53 by mutation has been implicated in a wide variety of human malignancies. Increased p53 levels have been detected in cultured skin fibroblasts from patients with Lynch Type 1 cancer family syndrome (non-polyposis colorectal carcinoma) (Kopelovich & De Leo, 1986). An inherited germ line mutation has been demonstrated in some (not all) of the affected members of Li Fraumeni syndrome families (clustering of sarcomas,

adrenocortical cancers, breast cancers, brain tumours and leukaemias) (Malkin et al., 1990; Srivastava et al., 1990).

RFLP analysis has demonstrated LOH on the short arm of chromosome 17 in the region of the p53 gene in 60% of breast carcinomas (Mackay et al., 1988; Devilee et al., 1989), 50-60% of ovarian epithelial carcinomas (Eccles et al., 1990; Russell et al., 1990), over 70% of osteosarcomas (Toguchida et al., 1989), 55% of astrocytomas (Fults et al., 1989), 63% of bladder carcinomas (Tsai et al., 1990), 75% of colonic carcinomas (Vogelstein et al., 1988), and up to 100% of small cell lung carcinomas (Mori et al., 1989) in some series.

Several monoclonal antibodies have been developed for the detection of both mutant and wild-type p53 gene products (Banks et al., 1986; Gannon et al., 1990; Midgley et al., 1992; Vojtesek et al., 1992). As the half life of the WT protein is short, and that of the mutated form increased, it is generally assumed that any p53 product detected immunocytochemically represents the mutant form (Lane & Benchimol, 1990). Mutations have also been detected by techniques employing sequencing of tumour-derived p53 DNA, mRNA or their PCR-amplified equivalents. By these methods, somatic mutations in the p53 gene have been found in a substantial proportion of tumours, and these may or may not be associated with allele losses in the 17p.13 region (Nigro et al., 1989; Chiba et al., 1990; Eccles et al., 1992).

It has been suggested that cervical carcinomas may segregate into two groups: those that are HPV and WT-p53 positive; and HPV negative tumours that possess mutated p53 (Crook et al., 1991b; 1992). In hepatocellular carcinoma, chemical mutagenesis

(attributed to aflatoxin exposure) has been associated with G to T transversions in codon 249 of the p53 gene in association with allele loss at 17p.13 (Bressac et al., 1991; Hsu et al., 1991).

Support for the role of p53 in tumorigenesis has also come from transfection assays in which WT- p53 gene transfected into cultured cells has been shown to suppress tumour growth, while mutated p53 gene has been shown to override this suppression (Baker, 1990a). Further, mutated p53 has been found to cooperate with ras to induce cellular transformation (Parada et al., 1984).

More recently, with the cloning of p53, transgenic mice carrying mutated p53 genes have been bred. They develop tumours, and their tumour profile resembles that in some Li Fraumeni families (Adams & Cory, 1991). Further, p53 null mice develop and reproduce normally but have a high incidence of tumours, particularly aneuploid tumours (Lane, 1992).

4.5. *PROPOSED MECHANISMS OF p53 TUMOUR SUPPRESSION*

Despite all the experimental data available on p53, its precise role in the cell cycle, and the mechanism by which it effects tumour suppression remain somewhat controversial. Newly synthesised p53 is located in the cytoplasm during G1, but shifts to a nuclear location during the early S phase (Shaulsky et al., 1990). It appears to have growth stimulatory properties in early S phase when DNA synthesis is occurring, but growth suppressor activity during G0 and G1. A temperature sensitive p53 mutant has also been identified, which can change its cytoplasmic location (WT conformation) to a nuclear location (mutant conformation adopted) at different temperatures. It has been shown that

UV light, irradiation and chemotherapeutic drugs that damage DNA, induce the accumulation of WT-p53 (Lane, 1992), and that its accumulation results in arrest of the cell cycle at G1.

All these findings have led to the currently favoured hypothesis which proposes a model of p53 as the "molecular policeman" or "guardian of the genome" (Lane, 1992), accumulating in the cell only when DNA damage occurs. Its accumulation is believed to effect tumour suppression by causing G1 arrest, thereby switching off replication, and allowing DNA repair to occur. Failure of DNA repair is believed to result in p53-triggered apoptosis. It thereby forbids replication of a DNA-damaged cell.

In tumour cells on the other hand, in which there is mutated p53, or in which DNA virus infection has resulted in sequestration (E1B, large T antigen) or rapid destruction (HPV E6) of p53, WT- p53 is unable to accumulate. G1 arrest therefore cannot occur, and cells with damaged DNA (more likely to develop mutations and chromosomal aberrations because of DNA instability) continue to replicate, and result in the evolution of malignant clones.

5. CYTOGENETICS & CHROMOSOMAL ABERRATIONS IN CERVICAL CANCER

In cervical carcinoma, reports of cytogenetic analyses have been few, and the number of tumours studied, when compared to cancers at other sites (eg. lymphomas and leukaemias which have been extensively studied) are relatively small. In general, the chromosomal picture has been extremely variable and complex, and complete karyotyping

has been accomplished in only a few cases. Triploidy and tetraploidy appear to be common, but no single cytogenetic abnormality has been consistently associated with this tumour type (Atkin & Baker, 1979; 1982; 1984).

As in many other types of neoplasia, chromosome 1 has been found to be involved in a non-random fashion, with aberrations comprising both numerical and structural rearrangements (Atkin & Baker, 1979; 1982; 1984). These aberrations which include isochromosomes, deletions, duplication, and associated translocations of both the long and short arms, have also been associated with other chromosomes, including chromosomes 3, 4 or 5, 6, 11, 13, 17, 18, and 21 (Atkin & Baker, 1979; 1982; 1984).

It has been suggested that chromosome 17-derived markers, which are frequently present in cytogenetic preparations from cervical carcinomas, may signify the importance of genes on this chromosome. Further, it has been postulated that their importance to the development of this cancer may lie in the loss of recessive genes on chromosome 17p (Atkin & Baker, 1989).

6. ONCOGENES & TUMOUR SUPPRESSOR GENES IN CERVICAL CANCER

What do we know at present about the role of oncogenes and tumour suppressor genes in cervical cancer? Evidence is still rather scanty but some interesting data have been generated.

6.1. *ONCOGENES AND CERVICAL CARCINOMA*

A study of the chromosomal location of cellular sequences flanking integrated HPV DNA sequences in cervical carcinoma cell lines and tumour tissue revealed that in some cases, sites of integration were within proximity of, or linked to oncogenes. In HPV 18-positive HeLa and C4-1 cell lines, the HPV DNA was found integrated within 40kb of the *c-myc* gene at chromosome 8q.24; and elevated steady state levels of *c-myc* RNA were found in these relative to other cervical cell lines (Durst et al., 1987). It is clear that *ras* gene mutations (particularly activating mutations in codons 12, 13 and 61 of H-*ras*, Ki-*ras* and N-*ras*) have been found in a variety of tumour types (Bos, 1989). Studies on oncogenes in cervical carcinoma have therefore focussed mainly on the *ras* and *myc* oncogenes.

6.2. *RAS ONCOGENE AND CERVICAL CARCINOMA*

In experimental models, HPV type 16 DNA has been shown to cooperate with the mutated c-Ha-*ras* gene in transforming primary cells (Matlashewski et al., 1988). Further, the transfection of activated H-*ras* to HPV 16- immortalised human cervical cells resulted in the development of cystic squamous carcinomas when these cells were injected into nude mice (Di Paolo, 1989).

In an immunohistochemical study, *ras* p21 protein overexpression was related to prognosis in cervical cancers, but the correlation was found to be dependant on the histological type (Sagae et al., 1989).

In one study, RFLP analyses of cervical carcinomas (90% of which were HPV positive) revealed LOH in tumour vs constitutional DNA at the c-Ha-*ras* locus in 36% of heterozygous tumours, but there was no correlation with tumour aggressiveness (Riou et

al., 1988a). In this study, 24% of advanced tumours were found to have somatic mutations at codon 12, affecting either one or both c-Ha-*ras* alleles. The presence of *ras* codon 12 mutation was found to be significantly ($p < 0.01$) associated with poor prognosis, and was associated with loss of the remaining c-Ha-*ras* allele in 40% of tumours (Riou et al., 1988a). In another study, analysis of Ki-*ras* mutation at codon 12 in tumours of the female genital tract failed to demonstrate mutation in the 4 cervical carcinomas examined (Enomoto et al., 1990). Riou et al. further reported that 100% of tumours containing mutated *ras*, and 70% of those with a deleted *ras* allele, also contained an amplified or overexpressed *myc* gene (Riou et al., 1988a & b).

The possibility of cooperation between c-*myc* and c-Ha-*ras* in the progression of cervical carcinoma has been suggested (Riou et al., 1988b). However, the results of molecular genetic studies on c-*myc* in cervical carcinoma remain somewhat controversial.

6.3. MYC ONCOGENE AND CERVICAL CARCINOMA

c-*myc* gene amplification and/or overexpression have been reported (Ocadiz et al., 1987; Riou et al., 1987). Riou et al. detected a 4-20-fold increase in c-*myc* expression in approximately one third of tumours examined, and this, together with c-*myc* amplification were significantly ($p = 0.001$) associated with more aggressive cancers - patients having an 8-fold increased incidence of early relapse. In another study, Ocadiz et al. reported c-*myc* amplification (some up to 60-fold) in 48% of tumour samples, with rearrangement of the gene in 90% (Ocadiz et al., 1987).

This was contrary to the findings of Riou et al. who detected no c-*myc* rearrangement, and Choo et al. who detected neither rearrangement nor amplification of c-*myc* in a study

on a Chinese population (Choo et al., 1989). The latter study also described altered levels of *c-myc* expression, thought to be related to HPV presence in some cervical cell lines.

Indeed the roles of these two oncogenes in cervical carcinogenesis have not been completely resolved and it remains uncertain whether oncogenes play any part in the development of this tumour.

6.4. *TSGs AND CERVICAL CARCINOMA*

Studies on the role of tumour suppressor genes (TSGs) in cervical carcinoma have so far been equally inconclusive.

The consistent finding of chromosome 17-derived markers in karyotypic analyses of cervical carcinomas led to the suggestion that the importance of chromosome 17 to the development of this cancer might lie in the loss of recessive genes on chromosome 17p (Atkin & Baker, 1989). Since then, LOH in primary cervical carcinomas has been reported for 100% of cases on chromosome 3 (in the region 3p.21 in a small series of 9 heterozygous cancers) (Yokota et al., 1989); 36% of cases at the *c-Ha-ras* locus on chromosome 11p (Riou et al., 1988a); 30% of cases on chromosome 11 using markers on both the short and the long arms (Srivatsan et al., 1991); and 14% of cases on chromosome 17p (Kaelbling et al., 1992).

Research on cervical carcinoma cell lines has suggested a role for genes on chromosome 11 (Kaelbling et al., 1986). Microcell transfer of a single copy of fibroblast chromosome 11 into tumorigenic HeLa cells converted them into a non-tumorigenic state (Saxon et al., 1986); and a putative TSG identified in HeLa cells has been mapped to the chromosome

11q.13 region (Srivatsan et al., 1991).

More recently the association between DNA viruses, in particular HPV, and the products of Rb and p53 TSGs has awakened a keen interest in the role, if any, of these two TSGs, but particularly of p53, in cervical carcinogenesis.

6.4. *Rb AND p53 TUMOUR SUPPRESSOR GENES IN CERVICAL CARCINOMA*

Few studies have examined the role of p53 or Rb in cervical carcinoma, but recent data have suggested an important role (Crook et al., 1991b & c; 1992). Human papilloma viruses, by contrast, have long been associated with cervical carcinogenesis (Bosch et al., 1989; zur Hausen, 1989a, 1991; Chang, 1990; Singer & Jenkins, 1991) although a definitive causative role has yet to be unequivocally established (Riou et al., 1990).

The HPV 16 and 18 E6 and E7 encoded oncoproteins (see Chapter 3) form complexes with WT-p53 (17p) (Banks et al., 1990; Werness et al., 1990; Scheffner et al., 1991) and pRb (13q) (Dyson et al., 1989; Banks et al., 1990; Scheffner et al., 1991) in vitro. It is believed that this complex formation confers growth advantage on the cell by inhibition of p53 (and Rb) activity (O'Rourke et al., 1990). Interaction between p53 and HPV 16 E7 has also been demonstrated in studies showing that expression of WT p53 suppresses the immortalising activity of HPV 16 E7, whereas mutated murine p53 potentially enhances its transforming activity in rodent systems (Crook et al., 1991d).

The formation of complexes between HPV E6 and p53 proteins has been shown (in vitro) to result in targetting of p53 for degradation through a ubiquitin-dependant proteolysis system (Scheffner et al., 1990; 1991) which ultimately leads to low (perhaps

undetectable) levels of p53 in the cell. It has further been shown that binding of p53 by HPV E6 proteins of benign HPV types (6 & 11) does not target p53 for degradation (Crook et al., 1991c). This is thought to be due to the fact that the C-terminal region of E6 which is conserved among all HPV types is important for p53 binding, whereas, the N-terminal sequences necessary to direct p53 degradation are conserved only between oncogenic HPV types.

Similarly, the HPV E7 proteins of both high and low risk genital type HPVs have been shown to bind pRb; but, types 6 & 11 bind with 20-fold and 5-fold lower affinities respectively than the E7 proteins of HPV 16 and 18 (Munger et al., 1989).

The HPV 16/18 E6 - p53 interaction has also been demonstrated *in vitro*, in that the half life of p53 was significantly diminished (2-4-fold reduction in p53 stability) in cells immortalised with HPV 18 E6 and E7 when compared to the non-transformed human foreskin keratinocytes. Immortalisation by E7 alone revealed a p53 half-life similar to that in non-transformed cells; but this was decreased significantly by supertransfection of an E6 gene (Scheffner et al., 1990).

Further experiments have revealed that HPV E6 abrogates p53 mediated repression of transcription in *in vivo* transient transfection assays, and that this inhibition was dependant on E6/p53 binding, but did not require degradation of p53 (Lechner et al., 1992). It has further been reported that, although some mutant p53 proteins bind HPV 16 E6 and are targetted for degradation, those that bind less well than WT-p53 are degraded less efficiently than WT-p53. It has therefore been suggested that the mutant form may exist in 2 forms - a WT-like conformation that binds avidly to E6 and is

targetted for degradation, and a mutant conformation that does not (Scheffner et al., 1992).

Much therefore remains to be resolved regarding the roles of these two tumour suppressor genes in cervical carcinogenesis.

CHAPTER 3

HUMAN PAPILLOMA VIRUSES (HPVs) AND CERVICAL CANCER

1. HPV VIRUS TYPES AND GENETIC ORGANISATION

1.1. *CLASSIFICATION OF PAPILLOMA VIRUSES*

Papilloma viruses are DNA viruses which have been implicated in the aetiology of diverse manifestations of epithelial neoplasia in higher vertebrates including rabbits, cattle, deer and man. The genus belongs to a family of viruses known as Papovaviridae, but each virus is named according to its natural host (eg. human papilloma virus (HPV) or bovine papilloma virus (BPV)).

Classification of papilloma viruses is further based on DNA sequence homology. This is determined by the degree of DNA cross- hybridisation in the liquid phase. To be designated as a new virus type there must be less than 50% homology to previously typed viruses. A viral DNA that hybridises to greater than 50% to a given virus type is considered as a subtype, and many viruses comprise subtypes which show minor differences in restriction enzyme cleavage patterns of their DNA. To date, over 70 different papilloma virus types have been identified, of which more than 50 are human papilloma viruses (Arends et al., 1990; Chang, 1990; Sousa et al., 1990).

The range of susceptible epithelia also differs among virus types so that papilloma viruses



can be further grouped according to the sites of associated lesions. It has been suggested that three papilloma virus families can be identified according to site specificity - genital, skin, and fibropapilloma viruses. The human papilloma viruses are confined to the group affecting genital and skin epithelia.

1.2. HPV STRUCTURE AND GENETIC ORGANISATION

The papilloma virus genome is composed of a single closed circular double-stranded DNA core. The virions comprise this DNA core enclosed within an outer capsid of viral protein. The viral genome is approximately 8,000bp in length, with a molecular weight of about 5.2×10^6 D. The capsid is composed of 72 capsomeres arranged in an icosahedral pattern which appears almost spherical on electron microscopy, and, in the mature particle, lacks an outer membrane envelope. Two types of protein comprise the capsid - the major highly cross-reactive proteins which serve as genus specific antigens; and the minor highly type-specific capsid proteins.

Despite a rather low degree of sequence homology between human and animal papilloma viruses, the linear organisation of all the papilloma viruses characterised to date has been remarkably similar, and has permitted the utilisation of BPV 1 as a model. The genome consists of at least 10 potential protein coding sequences or open reading frames (ORFs) all on the same strand; the other DNA strand contains only small unconserved ORFs, and is assumed to be non-coding (Arends et al., 1990; Chang, 1990; Sousa et al., 1990).

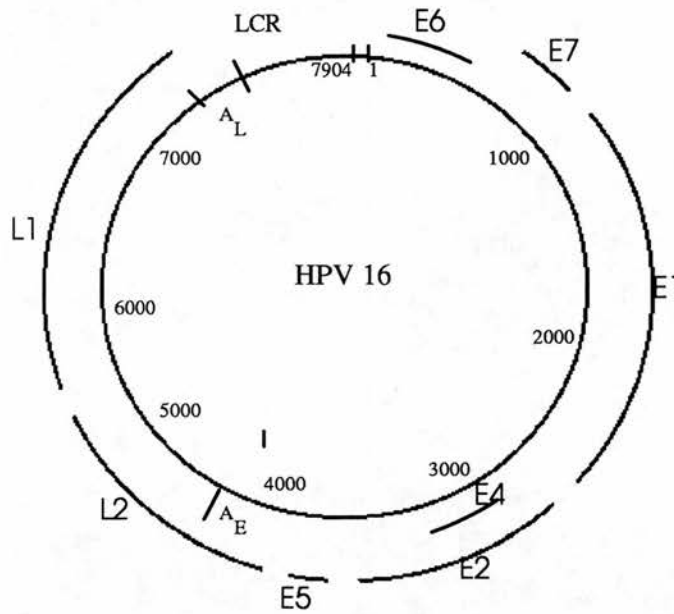
Functionally, the genome can be divided into 2 coding regions separated by a 0.4-1.0kb non-coding segment. The coding regions have been termed the E (early) regions of which

there are between 6 and 8, and the L (late) regions of which there are 2. In vitro transformation of rodent cells by BPV 1 has been used as a model system to assign specific functions to some of these regions.

E regions 1-8 contain the early genes E1-E8 which are important in viral replication and cellular transformation. The E6 and E7 ORFs code for the major transforming proteins and comprise 158 and 98 amino acids respectively. The L region contains the late genes L1 and L2 which code for structural proteins (L1 - major; L2 - minor) of the virion. The non-coding region is located between the end of L1 and the start of the E6 ORF and is termed the long control region (LCR) or the upstream regulatory region (URR). It contains transcriptional control sequences involved in the control of viral gene expression (see Fig. 2) (Arends et al., 1990; Chang, 1990; Sousa et al., 1990).

Fig. 2

GENOMIC ORGANISATION OF HPV 16



In this genomic map of HPV 16 deduced from the DNA sequence, the nucleotide numbers are noted within the circular maps, transcription proceeds clockwise, and the major open reading frames (E1-E7, L1 and L2) are indicated. A_E and A_L represent the putative polyadenylation signals for the early and late transcripts respectively. The viral long control region (LCR) containing the putative viral transcriptional and replication regulatory elements is noted. (Modified from Fig. 1, Howley, P.M. & Schlegel, R. (1988) The human papillomaviruses. An overview. *Am. J. Med.* **85** (Suppl 2A): 155-158).

1.3. HPV TYPES ASSOCIATED WITH CERVICAL CARCINOMA

Despite the identification of over 50 types of HPV, site specificity has dictated that only a few types namely types 6, 11, 16, 18, 31, 33, 35, 39, 42-44, 51 and 52 are associated with the human genital tract. Types 6, 11, 16, 18, 31, and 33 are most frequently isolated; and of these, only the latter four types have consistently been associated with malignancy in the cervix. However, most of the data on HPV in cervical cancer focusses on types 16 and 18, which together account for the HPV status of 80- 90% of cervical carcinomas and high grade CIN. In general, HPV types 6 and 11 have been associated with benign cervical neoplasia such as condyloma, and with lower grades of CIN (zur Hausen, 1989b).

2. BIOLOGY, LIFE CYCLE & MORPHOLOGICAL EXPRESSION OF HPV

2.1. BIOLOGY OF HPV INFECTION

An understanding of the biology and morphological expression of HPV infection is essential to the understanding of any HPV/cervical cancer association. While there is some overlap between lesions caused by different virus types, the variation in morphological expression of infection represents a complex interplay between HPV, the infected epithelial cell, host immunity and exogenous cofactors.

The factors which influence the process of containment of infection vs. regression or progression of lesions are poorly understood, but interactions between specific virus types and host cells, which determine the pattern of both host and viral gene expression appear important.

HPV infection may be permissive (allowing the appropriate microenvironment for productive viral replication) or non-permissive. Permissive infection results in the synthesis of episomal viral DNA, RNA and proteins, and the assembly and release of viral particles. The host may contain the infection as a latent infection ie carrier status, or vegetative viral replication may result in high copy numbers of the virus in superficial epithelial cells in lesions which are contagious but rarely neoplastic. There is viral DNA transcription, translation and virus specified protein synthesis. There is limited early gene expression; transforming proteins induce conducive host cell functions and regulatory proteins control viral gene expression. Viral DNA appears to remain episomal. It is only the differentiating cells of the stratum spinosum and stratum granulosum that permit productive viral replication (Arends et al., 1990).

Non-permissive infection refers to infection of non-permissive cells which lack the functions or appropriate microenvironment in which viral replication can occur. It leads in some instances to cellular transformation which is thought to result from viral integration into host DNA. It is believed that integration results in loss or alteration in viral or host gene function and leads ultimately to alterations in both genotype and phenotype of the host cells.

No specific integration sites have yet been identified in the host genome (Dalglish, 1991; zur Hausen, 1991), but the opening of the viral circular DNA shows remarkable specificity during integration. The opening usually occurs within the 3' end of E1 or the 5' end of E2 thereby disrupting the genome in such a way that the E2 transactivator gene is no longer functional (Arends et al., 1990). This leads to loss of viral self-regulation, with

the viral transforming genes coming under the control of host cellular genes.

In non-productive infection, there is pronounced expression of the early ORFs of the viral genome, which may result in increasing basal cell hyperplasia, decreasing maturation of host cells, and the gradual development of an aneuploid chromatin and dysplastic morphology. Viral copy number per cell is low, late gene products are diminished, and there is an association with the increased expression of viral transforming gene (E6 & E7) products.

Viral DNA is always present in episomal form and in multiple copies in benign lesions, but almost always integrated into the host genome in carcinomas (Pfister, 1987).

2.2. THE LIFE CYCLE & MORPHOLOGICAL EXPRESSION OF HPV INFECTION

The cycle of HPV infection can be divided clinically into three phases - a latent phase, an active growth phase, and a host response phase. Infection may be contained, or lesions may regress or progress at any phase in the cycle. However, the duration of each phase shows extremely marked variation between individuals and the potential for progression varies between virus types.

Exposure to the virus, presumably at intercourse, results in inoculation of the virus at sites of microtrauma in the genital epithelium. The virus is directly inoculated or transported into basal cells where it is established in the latent phase as an episome. Basal cells are non-permissive for HPV maturation. However, during transit from the basal layer to the surface, the keratinocyte undergoes a series of complex changes which confer

permissiveness. This leads to an incubation phase during which the viral numbers per cell increase significantly by viral replication, and which persists for long periods (usually between 6 weeks and 8 months) without disease expression. The infection may be contained at this stage resulting in host carrier status.

Alternatively, the active growth phase with focal areas of active expression of viral infection seen as epithelial and capillary proliferation may follow. This itself has two stages. The first of these stages is one of subclinical, minimally expressed low grade cervical epithelial atypia which may appear as aceto-white and/or vascular changes colposcopically, but histologically appears normal or shows minimal basal hyperplasia and dyskeratosis. Results of immunocytological studies indicate that virion assembly is occurring at this stage, consistent with an infection in evolution, and suggest that there is a potential for infectivity. Progress may be arrested at this stage and in some cases disease will spontaneously regress.

Alternatively, disease may progress to a more fully expressed infection with a markedly increased risk of neoplastic transformation. This established active growth phase is characterised by rapid epithelial and vascular proliferation and results in the production of warty condylomata or "flat HPV lesions" (aceto-white plaques). To this stage, high copy numbers occur in superficial epithelial cells and lesions are contagious but rarely neoplastic. Both lesions are predominantly due to "low risk" HPV types (6 & 11), and exhibit a tendency towards spontaneous regression over a variable time period.

However, as a small proportion are due to "high risk" types (16, 18, 31, 33, 35, 39); at least 20% of women with condyloma will have coexistent CIN on histology; and although

two thirds of flat HPV lesions will regress spontaneously or therapeutically, one third persist as refractory atypia, become more extensive and appear to be at high risk of neoplastic transformation (reviewed by Campion, 1989).

These lesions are regarded as markers for increased risk of potential for neoplastic or malignant transformation. Those with increased potential are thought to be the result of non-productive viral infection predominantly with HPV 16, 18, 31,33,35 and 39. In such cases viral copy number per cell is likely to be low, and viral DNA may integrate into the host cell genome. Indeed, though such infection may be less contagious, it is thought to have a much greater potential for neoplastic transformation, a risk which is diminished only by complete and adequate destruction of the cervical transformation zone (TZ). This is thought not only to destroy the "high risk" TZ epithelium, but also to decrease the virus load, thereby permitting the host immune system to contain the infection.

HPV lesions are associated with marked changes in humoral and cell mediated immunosurveillance which normally protect against neoplasia. Indeed it has been shown that the relative paralysis of the system which occurs in association with flat HPV lesions persists in the neoplastic epithelium. The host response phase occurs over a period of 9 months to several years, is modified by exogenous cofactors (eg. seminal fluid, cigarette smoking), and hence shows variability between individuals (Campion, 1989).

The biological potential of cervical HPV infection is therefore dependent on several host, viral and exogenous factors, interaction of which may result in containment or persistence of the virus, or in disease persistence, regression or progression to neoplastic transformation.

3. METHODS OF DETECTION AND IDENTIFICATION OF HPV

Studies on HPV have been hampered by the inability to grow these viruses in cultured cells in vitro, or to infect laboratory animals. Nevertheless, several methods have been developed for their detection in clinical material which largely consists of cytological smears and tissue biopsy specimens.

The biological behaviour of the virus involving variations in virus copy number per cell, viral gene expression and viral antigen expression in different phases of its life cycle dependent on the infected epithelium or lesion, has posed problems in the development of a reliable, sensitive and specific test that could be applied to all specimens. The development of type specific tests and tests able to identify the status of the virus within the cell (integrated vs. episomal) have posed even greater challenges.

3.1. *CYTOLOGICAL AND HISTOLOGICAL DETECTION OF HPV*

HPV viral changes in epithelia can be identified cytologically and histologically; but their absence is notoriously unreliable as an estimate of the absence of HPV DNA, or as a measure of exposure to HPV.

Cytopathic effects of HPV infection including koilocytosis, dyskeratosis and multinucleation can be identified in cervical smears. Koilocytosis, seen also on histological sections, is widely considered as the diagnostic hallmark of HPV infection, and is a result of late gene expression of the viral genome. The koilocyte is a mature squamous cell characterised by a large perinuclear cavity. The nucleus, often double but sometimes multiple, is hyperchromatic and twisted in appearance. The chromatin is

smudged and there is no nucleolus. It is a dead or dying cell. Hypergranulosis (prominence of keratohyaline granules of keratinising squamous epithelium) is a common feature.

CIN, commonly seen in association with these features in cervical biopsies, is a pre-neoplastic change comprising disorders of differentiation of mostly immature squamous epithelium. Nuclear enlargement, and altered chromatin patterns are common, and abnormal mitotic figures may be seen. Maturation of the squamous epithelium is often disorderly and involves all epithelial layers. However, the basement membrane remains intact. These are all changes which can be, but are not necessarily, associated with HPV infection, and are therefore not diagnostic of it.

There are basically four direct methods for diagnostic detection of HPV infection in clinical material - electron microscopy, immunocytochemistry, nucleic acid hybridisation techniques, and DNA amplification techniques.

3.2. HPV DETECTION - ELECTRON MICROSCOPY & IMMUNOCYTOCHEMISTRY

Electron microscopy has not gained widespread use as it requires both elaborate and time-consuming preparation of specimens and expensive equipment. Further, it is a relatively insensitive technique when compared with nucleic acid hybridisation and DNA amplification techniques, and cannot differentiate between virus types.

Immunocytochemistry depends on the detection of HPV common capsid antigen by polyclonal antibody. It is therefore not type specific, but serves as an indicator of late gene expression of the virus particles, and of potential infectivity. This method can be

used on formalin-fixed, paraffin-embedded tissue, but one of its disadvantages lies in the fact that only well differentiated cells appear to permit viral antigen expression. This effect arises because the protein antigen targeted by most antibodies is accessible only during virion assembly. Hence, many highly infective lesions such as condyloma, with many formed virions per cell, and HPV infected high grades of CIN and invasive neoplasia rarely contain antigen, and are often falsely immunocytochemically negative.

3.3. MOLECULAR BIOLOGICAL TECHNIQUES FOR DETECTION OF HPV

Molecular biological methods - nucleic acid hybridisation and polymerase chain reaction techniques - are the only sensitive and type specific methods available for direct detection of HPV in tissues. These methods both detect viral genetic information regardless of the state of differentiation of the cells; and variations of these techniques can be applied to fresh tumour or cells, frozen material, or to formalin-fixed paraffin-embedded tissue.

A wide variety of molecular hybridisation tests is available. They vary in sensitivity, specificity, and requirements for sample preparation. Essentially, HPV genetic information in a clinical sample is detected by probing for either DNA or RNA, and may involve DNA:DNA, DNA:RNA, or RNA:RNA interactions under different stringency conditions. Probes are labelled by incorporation of either radiolabelled, biotinylated, or digoxigenin-labelled DNA or RNA nucleotide precursors in synthetic reactions.

3.3.1. Southern Blotting Hybridisation

Southern blotting hybridisation relies on the use of radiolabelled type specific HPV probes, which recognise and hybridise to DNA sequences (derived from digested and size fractionated genomic DNA extracted from clinical specimens) which have been

transferred to and immobilised on nylon membranes. DNA from approximately 10^6 cells is required, and on average, it will detect 0.1 to 1.0 viral genomes per cell. Types and subtypes can be detected on the basis of their characteristic restriction enzyme cleavage pattern, and this is currently the only method of providing information on the episomal or integrated state of viral sequences.

Though labour intensive, and expensive in time, fastidiousness, equipment and reagents, it remains the most sensitive and specific of the molecular hybridisation techniques, and is regarded as the "gold standard" by which other nucleic acid hybridisation techniques are judged. The use of radioactivity perhaps limits its usefulness in routine clinical and epidemiological settings.

3.3.2. *Reverse Southern blotting and Dot blot hybridisation*

Other nucleic acid hybridisation techniques include reverse Southern blotting, in which labelled cellular DNA is used as a probe against a bank of HPV types fixed to a membrane. Although a useful technique, it suffers from diminished sensitivity.

Dot blot hybridisation involves spotting of concentrated HPV DNA or RNA directly onto a small area on a membrane, prior to probe hybridisation. It is less time consuming than Southern blotting, and its sensitivity compares well. However, specificity is a problem, in that cross hybridisation occurs, and weak positives are difficult to distinguish from background. Results are not always entirely reproducible, and difficulty sometimes arises from the inadequacy of specimen collection in cervical smears.

3.3.3. *In situ hybridisation techniques*

Filter in situ hybridisation is a further variation on this theme, similar to dot blots, but not involving a DNA purification step. Like dot blot hybridisation, it requires experience in performing the technique as well as in the interpretation of the results. Low sensitivity, especially in high grade lesions, and false positives resulting from cellular or extracellular debris pose further problems.

In situ hybridisation uses labelled DNA/RNA probes to detect the presence of viral sequences within cells of histological tissue sections, which are fixed to specially treated (Tesda coated) glass slides. It is technically difficult, time consuming, and lacking in sensitivity especially for high grade lesions. It requires some cells at least to contain 20 - 50 viral genomes.

These methods are all dependent firstly on probe fidelity in annealing to test nucleic acids. Secondly, and perhaps more importantly, the sensitivity and specificity in detecting HPV DNA seem to differ according to whether the sample is from low or high grade CIN, invasive carcinoma, or normal tissue. It has been suggested that methods involving DNA extraction (Southern and dot blots) are more sensitive in specimens like tumours with low copy numbers/cell, while in situ methods are more sensitive when the copy number is high in relatively few cells eg in condylomata or in normal cervical epithelium. All of the above techniques are reviewed by Lancaster & Norrild, (1989).

3.3.4. *Polymerase chain reaction (PCR) for detection of HPV*

Polymerase chain reaction is perhaps the most novel and reliable method for HPV detection. It supersedes Southern blotting techniques in sensitivity and specificity, and

involves the use of type specific oligonucleotide primers (that are complementary to opposing DNA strands, a short 100 - 200 nucleotides apart), and DNA polymerase, in temperature controlled cycles of denaturation, annealing, and extension, for the exponential amplification of these specific short DNA sequences.

Its advantages lie in its ability to utilise DNA derived from fresh, frozen, or fixed paraffin-embedded tissue; its requirement for small amounts of tissue for analysis - theoretically its limit of sensitivity would be one copy of a viral genome per sample; its cost effectiveness in terms of time and labour, though less so in terms of reagents and equipment; and its great potential for automation. One of its drawbacks is the potential for contamination of samples and equipment, and scrupulous attention must be paid to technique (Cornelissen et al., 1989; Editorial, 1989; Lancaster & Norrild, 1989; Arends et al., 1991).

Given the various methods which have been available for detection, it is not surprising that the results of clinical and epidemiological studies attempting to demonstrate the prevalence or aetiological association of HPV infection with cervical carcinoma, have, because of differences in reliability, sensitivity, and specificity of techniques employed, yielded inconsistent and often conflicting results. An attempt is made in the following section to summarise some of the evidence for and against this association.

4. EVIDENCE FOR HPV ONCOGENICITY

There is a wealth of evidence implicating the oncogenicity of papilloma viruses, and an equally large body of evidence suggesting a major aetiological role for HPV in the

development of cervical neoplasia. Papilloma viruses appear to exhibit three common features relevant to their oncogenicity. Firstly, there are differences within species in the oncogenic potential of virus types; secondly, a long latent period (up to several years) between infection and malignant transformation is characteristic; and finally, exogenous cofactors, such as chemical or physical carcinogens, seem to be necessary for the viral infection to effect malignant transformation.

4.1. *ANIMAL MODELS*

Recognition of the oncogenic potential of papilloma viruses has come largely from observations of naturally occurring cancers in animals.

One of the earliest observations, was that 75% of cotton tail rabbit papilloma virus (CRPV) or Shope virus-induced papillomas in domestic rabbits progressed to malignancy. Further, it was noted that the incidence and rate of progression of these lesions could be enhanced by repeated application of a chemical carcinogen, methylcholanthrene or tar (Rous & Kidd, 1938).

More recently similar observations have been made in cattle in the Scottish Highlands. The malignant progression in these animals of BPV type 4-induced alimentary tract papillomas is increased by the synergistic effect of chemicals, particularly quercetin (a carcinogen) and azathioprine (a potent immunosuppressant), both of which are present in bracken fern which the animals ingest. The effect is not only an increase in the numbers of alimentary tract cancers, but also an increased incidence of other bovine cancers such as bladder cancers (reviewed in Chang, 1990).

The results of in vitro studies employing animal viruses have supported these findings. BPV 1 has been successfully used to transform mouse cells such as NIH 3T3, and transformed cells exhibit characteristic features, including altered morphology, serum and anchorage independent growth, and tumorigenicity in nude mice. Cancers of the alimentary tract have also been experimentally induced by BPV 4 infection of bracken-fed, and azathioprine-suppressed animals (Jarrett, 1987).

4.2. ASSOCIATION BETWEEN HPV AND NEOPLASIA IN MAN

The earliest evidence for an oncogenic association of HPVs came from observations in patients with epidermodysplasia verruciformis, in whom almost all HPV types have been identified, but who show a particular susceptibility to the development of cutaneous cancers in lesions infected mostly by HPVs 5 and 8. Sun exposed areas of skin are particularly vulnerable, and infection with other HPV types is unlikely to progress to malignancy (Orth et al., 1978).

Since then, HPVs have been detected in benign and malignant lesions of the upper digestive and respiratory tracts in man, and more recently, even in normal, benign and malignant colonic epithelium (Kirgan et al., 1990).

Of greater relevance, has been the growing interest in, and the increasing evidence for the involvement of HPV types 16, 18, 31, and 33 in the genesis of cervical cancer. Support for this association, and for their oncogenic potential has essentially come from two sources - firstly, from clinico-epidemiological observations and studies; and secondly, from laboratory-based experimental studies.

4.3. HPV AND CERVICAL CANCER - CLINICO-EPIDEMIOLOGICAL EVIDENCE

Most of the data that have been generated from clinico-epidemiological type studies on cervical carcinoma, are consistent with the hypothesis that HPVs are an important factor. It is known that HPVs are epitheliotropic - inducing proliferative epithelial lesions of skin and mucosae in man. The suggestion that HPVs could be the sexually transmitted agent involved in cervical carcinogenesis was formally put forward by zur Hausen in 1976. Since then, the general conclusions from numerous prevalence studies have been several: most cervical carcinomas harbour HPV DNA; in-situ hybridisation studies have localised the viral genome within tumour cells; and the persistence of viral DNA and RNA in carcinomas may implicate a direct role in conversion to, and maintenance of, the malignant state (Bosch & Muñoz, 1989).

Certain specific types of HPV have been consistently associated with cervical neoplasia - types 16 and 18 are the most frequently detected; women with cervical neoplasia have HPV 16 and 18 detected in their cervical cells more frequently than women with normal cervixes; and types 6 and 11 are seldom found in invasive disease, but occur more commonly in CIN lesions than in the normal cervix (Stoler et al., 1992).

HPV DNA can be identified in 80-90% of cases of CIN regardless of its grade - histological evidence of HPV infection is reported in over 80% of cervical biopsies (Jenkins et al., 1986); and similar cytopathic effects have been detected in normal epithelium or lower grade CIN lesions in the epithelium surrounding 90% of squamous cervical cancers (Reid et al., 1982). DNA of the same HPV type is usually identified from all lesions (in a given individual) regardless of grade, but the prevalence rates of HPV 16 and 18 increase with the severity of the lesions.

These observations could all imply a role for these viruses in the sequential steps of a multi-stage process. Further, viral DNA is often found integrated into the host cellular DNA in CIN 3 and invasive cancers, suggesting that integration may have an initiating role, leading to loss of viral self regulation, with its transforming genes coming under the influence of host cellular genes. Finally, resected specimens of cervical cancers containing "high risk" HPV 16 and 18 have shown alterations in function of cellular oncogenes.

4.4. *HPV AND CERVICAL CANCER - EXPERIMENTAL EVIDENCE*

Laboratory-based experimental studies have been of two types. Some have looked at the in vitro transforming capacity of the virus and its component genes; while others have examined viral status, viral gene, RNA, and protein expression, and their putative effects on the host cell genome, in resected specimens of cervical neoplasia or cell lines derived from them.

Transforming activity for different HPV types, including types 1, 5, 8, 11, 16 and 18 has been demonstrated in vitro. Similar to BPV 1, cloned HPV viral DNA has been introduced into rodent cell lines successfully to effect transformation defined by altered morphology, loss of serum and anchorage dependence, and tumorigenicity in nude mice. The transforming efficiency of HPVs is lower than that of their bovine counterparts, but transforming activity has confidently been assigned to the E6 and E7 ORFs. It is still uncertain, however, whether differences exist between the in vitro transforming capacity of HPVs differing in in vivo oncogenic potential (Arends et al., 1990; Chang, 1990).

Immortalisation has been demonstrated in human foreskin keratinocytes that have been

transfected with HPV 16, 18, 31, or 33, but not with types 6 or 11; and when allowed to stratify into multilayer epithelia, those transfected with HPV 16 show characteristics of CIN3. Immortalised cell lines contain integrated, transcriptionally active viral genomes but are not tumorigenic *in vivo*; the viral DNA remains episomal in those transfected with HPV types 6 or 11, and their lifespan is the same as that of non-transfected cells (Woodworth et al., 1989).

Transfection of NIH 3T3 (cultured fibroblast) cells with HPV 16 DNA results in stimulation of their growth properties, but prolonged incubation results in recognisable morphological changes in approximately 1 cell per 1000, with no apparent alteration in the state or expression of the viral DNA - a change thought to result from a second, probably mutational, cellular event (Noda et al., 1988). Experiments on primary rat cells have identified a cooperative effect between HPV 16 and the activated ras oncogene (Matlashewski et al., 1988). More recently, *in vitro* experiments have demonstrated binding of HPV 16 and 18 E6 and E7 genes to the protein products of the p53 and Rb tumour suppressor genes respectively. The rapid degradation of E6-bound p53 by a ubiquitin-dependant pathway has already been discussed.

Several studies have examined viral expression and viral interaction with host cellular genes in primary cervical tumours and cervical carcinoma cell lines. In all HPV 16 or 18 positive cell lines (eg. CaSki, SiHa, HeLa), and in most cervical cancers and high grade CIN lesions, the viral genome has been detected as integrated into the cellular DNA (Durst et al., 1985). It has been found that viral RNA transcripts are invariably present, confirming the transcriptionally active state of the viral DNA in CIN 3 and invasive cancer.

The products of the E6 and E7 ORFs are the viral proteins most consistently detected in HPV-positive cervical carcinoma cell lines and in cells transformed in vitro. These have been shown to have transforming properties, and may be required for the initiation and maintenance of the transformed state. Tissue specific constitutive enhancers have been identified in the regulatory regions of HPV 16 and 18. It has further been shown that their promoting influence on expression of E6 and E7 ORFs is not affected by the disruption of the E2 region, which accompanies viral integration, and may imply an important E6 and E7 enhancing role in cervical carcinogenesis (reviewed in Shah & Gissmann, 1989).

Although the site of cellular integration is usually variable, a single HPV integration site of HPV 16 in the SW 756 cell line has been identified as a heritable fragile site on chromosome 12 in the 12q.13 region. Studies on the sites of integration of HPV 18 in the host cell genome of cervical carcinoma derived HeLa and C4-1 cell lines have demonstrated integration at chromosome 8q.24 in the region of the *c-myc* oncogene. The detection of elevated *myc* mRNA in both cell lines has suggested that activation of this cellular oncogene may occur by integration of viral promoter/enhancer sequences (Durst et al., 1987). Resected HPV positive specimens of CIN and invasive carcinoma, as well as cell lines have demonstrated alterations in levels of host cellular oncogene expression.

Studies on HeLa (HPV 18 positive) cell/normal fibroblast hybrids demonstrated loss of both tumorigenicity in nude mice and HPV 18 expression in vivo. This effect was reversed by the progressive loss of chromosomes (particularly chromosome 11) from the resultant hybrid. This suggested that there may be some cellular genes that suppress the oncogenic functions of integrated viral HPV 18 DNA (Kaelbling et al., 1986; Saxon et

al., 1986).

All of these observations of associations of HPV with cervical cancer, and demonstrations of their oncogenic potential in vitro and in vivo, are balanced by data which suggest that HPV cannot be the only factor involved in malignant transformation in the cervix. This has led to the proposal of several putative multistep mechanisms of carcinogenesis, some of which will be discussed in the following chapter.

5. EVIDENCE AGAINST A CENTRAL ROLE FOR HPV IN CERVICAL CANCER

Despite several lines of evidence implicating a major role for HPV (particularly types 16 and 18) in the aetiology of cervical cancer, there are others to the contrary, which suggest that, if HPVs are involved, they are not the only factors.

The true prevalence of HPV in the general population is unknown, but recent studies indicate that it may be a lot higher than hitherto appreciated. HPV 16 and 18 DNA has certainly been isolated from cervical smears of patients with clinically and histologically normal cervixes (Reeves et al., 1987). It has also been isolated from normal cervical epithelium in patients with genital cancer, and as previously mentioned, has recently been detected even in normal colonic mucosa (Kirgan et al., 1990). Further, HPV negative cervical carcinomas have been reported repeatedly.

These findings would suggest that HPV may merely be a passenger, and that it may not play a significant role in cervical cancer causation (McNab et al., 1986). Indeed, the high

prevalence of latent HPV infection would indicate that HPV alone may not be sufficient for neoplastic transformation. This is supported by the finding that cells immortalised by HPV in vitro (even "high risk" types) are not oncogenic in vivo, implying that a second event or factor may be required (Di Paolo et al., 1989).

CHAPTER 4

AN INTEGRATION OF IDEAS; AIMS OF THIS STUDY

1. A MULTISTEP MECHANISM FOR CERVICAL CARCINOGENESIS?

In view of the previously described synergism between papilloma viruses and chemical or physical carcinogens, which appears to be necessary for malignant transformation in animals, it is no surprise that there is evidence to suggest that something more than HPV infection may be required for cervical carcinogenesis. Furthermore, the long latent period, and the cofactor requirement imply a multistep mechanism of carcinogenesis, with at least a second (or perhaps multiple) event(s) being required in addition to HPV infection.

In keeping with this theory are the findings of altered oncogene function, particularly *c-myc* amplification or overexpression, and *c-Ha-ras* amplification, deletion, mutation, or combinations thereof, which have been described in HPV positive (usually advanced) cervical carcinomas and cell lines (Riou et al., 1988b). Alterations in tumour suppressor gene function have also been proposed as events in this process.

There have been reports of loss of p53 function through deletion or mutation in some cervical cancers (Crook et al., 1992; Kaelbling et al., 1992). Further, the discovery of interactions between HPV 16 and 18 E6 and E7 and the TSGs p53 and Rb respectively, which result in their instability, degradation, and ultimate loss of function, has focussed

interest particularly on these two tumour suppressor genes and their role(s) in cervical carcinogenesis. Loss of function of other TSGs (some perhaps as yet unidentified) has also been proposed as a contributory factor.

It has been proposed that viral integration may effect these changes in behaviour or function of host cell oncogenes or TSGs. However, the mechanisms by which any of these factors contribute remain largely unknown. Whether oncogene activation, TSG inactivation, or a combination of both serve as primary events resulting from the effect of chemical mutagens in cigarette smoke or seminal fluid, with viral and other infections being incidental or secondary; or whether they are important, singly, or in combination, as events which occur secondary to the primary initiating event of viral infection remains controversial. It nonetheless remains plausible (even likely) that a combination of all these events is necessary for the multistep process of malignant transformation in the cervix.

2. AIMS OF STUDY

This study set out to examine the role of commonly implicated tumour suppressor genes (TSGs) in cervical carcinogenesis; to shed further light on the prevalence of p53 mutations in normal preinvasive and invasive cancerous cervix and on its relationship, if any, to the HPV status of these lesions; to examine for ras oncoprotein expression in normal, premalignant and malignant cervical epithelium; and to correlate these findings with the DNA ploidy of tumours examined, and ultimately with patient outcome. The study was carried out on samples obtained from a South-East of Scotland based population.

In an attempt to learn whether known TSGs are involved in the genesis or progression of cervical carcinoma, or whether the presence or integration of HPV into host DNA influences the pattern of molecular lesions, cases of cervical carcinoma were examined for loss of heterozygosity using several polymorphic DNA probes, which hybridised in the vicinity of putative oncosuppressor gene loci identified in RFLP analyses of other common solid human tumours. The HPV status of each tumour was examined by HPV type specific polymerase chain reaction (PCR) and, where possible, an attempt was made to relate the presence or integration of HPV sequences to the allele losses observed. The intention was to identify any consistently deleted regions of the genome, so that these regions could be analysed in specimens of preinvasive (CIN) phases of cervical carcinoma to determine at what stage in disease progression these aberrations occurred.

To shed further light on the prevalence of p53 mutations in cervical carcinomas, and to determine its relationship to HPV status, and stage or histological type, the four "hot spot" (Nigro et al., 1989) regions of the p53 gene were screened for point mutations in a series of cervical tumours using polymerase chain reaction (PCR), followed by denaturing gradient gel electrophoresis (DGGE). These results were compared with those of immunocytochemistry, and were correlated with the HPV status of lesions. Any mutations identified were further characterised by sequencing.

An extensive series of cases including biopsy specimens from normal, premalignant and malignant cervix was examined for the presence of accumulated (presumed mutated) p53, using an immunocytochemical method. Where possible, correlations were made between these results and the p53 status of the carcinomas as determined by PCR/DGGE, their

HPV status, and their 17p allele loss status.

A similar series of cases was examined for immunocytochemical evidence of ras p21 expression, to identify any differences between normal cervix, preinvasive and invasive carcinoma in their expression of this oncoprotein product.

Finally, the DNA ploidy of a series of invasive carcinomas was analysed by flow cytometry, and the results correlated with the other parameters analysed, as well as with patient outcome.

PART II

MATERIALS AND METHODS

The composition of reagents and solutions mentioned in this Section is given in Appendix 1.

PART II MATERIALS AND METHODS

CHAPTER 1 DNA ANALYSIS

1. Tissues
2. DNA extraction

CHAPTER 2 RFLP ANALYSIS

1. Tissues
2. DNA digestion
3. Agarose gel electrophoresis
4. Preparation of gel for Southern blotting
5. Southern blotting
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CHAPTER 3 DNA FINGERPRINTING

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CHAPTER 4 HUMAN PAPILLOMAVIRUS (HPV) ANALYSIS

1. Polymerase chain reaction (PCR) for HPV

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CHAPTER 5 DETECTION OF p53 MUTATIONS BY POLYMERASE CHAIN REACTION (PCR) FOLLOWED BY DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE)

1. Tissues
2. p53 PCR primers
3. p53 PCR technique
4. Gel electrophoresis and detection by UV transillumination
5. Denaturing gradient gel electrophoresis of PCR products
6. Analysis of tumour DNA by sequencing of p53 gene regions

CHAPTER 6 DNA PLOIDY ANALYSIS BY FLOW CYTOMETRY

1. Tissues
2. Preparation of samples for analysis
3. Sample analysis
4. Interpretation of tumour ploidy from DNA histograms

CHAPTER 7 IMMUNOHISTOCHEMISTRY

1. p53 Immunohistochemistry
2. ras p21 Immunohistochemistry

CHAPTER 1

DNA ANALYSIS

1. TISSUES

Paired tumour/blood samples were obtained from 55 patients with clinically overt and histologically confirmed invasive carcinoma of the cervix. The carcinomas were all of FIGO stage greater than or equal to Ib. Patients were seen prior to treatment by either Wertheim's hysterectomy or radiotherapy (usually the day before therapy). At this time, their informed consent to participate was obtained; and a 30ml sample of peripheral venous blood was taken from each patient into 3 x 10ml lithium heparin- coated tubes. The blood was gently agitated and kept at room temperature until lymphocyte separation was performed (normally within 1-12 hours).

At the time of surgery, or examination under anaesthesia (EUA) prior to radiotherapy, tissue samples were collected fresh from the operating theatre.

In the case of those undergoing Wertheim's hysterectomy, the uterus and cervix were collected onto crushed ice immediately on resection, and transported without delay to the Pathology Department. One to three samples of tumour were then carefully selected by a pathologist for use in this research.

Tumour samples varied in size, but were usually of the order of 0.3-0.5cm³, and depended on the size of the tumour. They were generally excised from the centre of the lesion, so as not to disrupt the tumour margins (which might jeopardise the accuracy of histological staging and diagnosis). Tumour samples were placed into individually labelled Eppendorf tubes and were 'snap' frozen in liquid nitrogen prior to storage at -70°C.

Specimens obtained from patients undergoing EUA prior to radiotherapy were taken using a Schumacher biopsy forceps. A minimum of 1 and a maximum of 3 samples were obtained from each patient, and were usually smaller than those obtained at hysterectomy. Each sample was placed into a labelled Eppendorf tube and immediately 'snap' frozen in liquid nitrogen.

In all cases, the presence of tumour tissue was confirmed by a gynaecological pathologist, who also estimated the ratio of tumour to stroma on frozen section microscopy. Any non-cancerous tissue was trimmed from the specimen before use in DNA analysis; and specimens with less than 70% carcinoma on frozen section were discarded. Frozen specimens were stored at -70°C until required.

Of the 30mls of peripheral venous blood obtained, lymphocytes were separated from 20mls for DNA extraction; while the remaining 10mls were used to establish cell lines by EB virus transformation of lymphocytes as previously described (Diehl et al., 1964).

2. DNA EXTRACTION

2.1. *PREPARATION OF BLOOD LYMPHOCYTES*

20 ml samples of heparinised blood from each patient were kept at room temperature and used within 12 hours of collection for separation of lymphocytes. The 2 x 10ml samples were centrifuged at 2500 rpm (95% brake) for 5 mins. Plasma was then pipetted off and discarded; and phosphate buffered saline (PBS) was added to each tube, to a total volume of 10mls.

This mixture was then carefully layered onto 10mls of Ficoll Hypaque in a sterile universal container. Centrifugation was performed at 2500 rpm (95% brake) for 15 mins; following which, the visible lymphocyte band (middle layer) was pipetted off into another sterile universal container; the rest being discarded. Dulbecco's PBS was added to each universal container, to a total volume of 20 mls, to wash the lymphocytes; and the suspension was then centrifuged at 2500 rpm (95% brake) for 5 mins.

The supernatant was poured off and 3ml of lysis buffer were added to the cellular deposit which remained. It was gently agitated and stored at 4°C, usually overnight, until DNA extraction.

2.2. *PREPARATION OF TUMOUR TISSUE*

Each sample of cervical tumour tissue was removed from storage, onto a tissue culture dish, where it was allowed to thaw until it was just sufficiently defrosted to allow cutting with a blade. A hand-held single-edged razor blade was used to mince the tumour tissue finely.

The minced tissue was then transferred to a hand-held tissue homogeniser (Wheaton, USA) and 2mls of lysis buffer were added. The tumour was homogenised by several minutes of repeated "mortar and pestle"-type crushing and the resultant suspension was transferred to a sterile universal container. A further 1ml of lysis buffer was added, and the suspension stored overnight at 4°C, awaiting DNA extraction.

Methanol and water were used to clean the homogeniser between cases.

2.3. PREPARATION OF DNA

DNA extraction was accomplished in all cases by using an automated system. Using a sterile pastette, the lysis buffer suspensions were each transferred from the universal container to individual glass columns in the DNA extractor (Applied Biosystems). Here the cell suspensions were subjected to cycles of RNAase and Proteinase K treatment, followed by repeated cycles of phenol-chloroform extraction, and a final step of ethanol precipitation. The extraction procedure was completed in 4-5 hours. At the end of the procedure, dried threads of DNA were retrieved from the surface of small discs of filter paper, within the collecting receptacles, at the exit end of each glass tube. The DNA extractor was automatically subjected to methanol washes between runs, in order to avoid cross contamination between samples.

DNA threads removed from filters, were each placed into sterile labelled Eppendorf tubes, with 100µl of 10T 0.5E buffer, and stored at 4°C. They were allowed 1-3 weeks for complete dissolution, before the DNA concentration was determined by spectrophotometry.

2.4. DETERMINATION OF DNA CONCENTRATION

10 μ l samples of DNA were each mixed thoroughly with 740 μ l of sterile distilled water. The spectrophotometer (Cecil CE 594) was calibrated using a sample of sterile distilled water in each of the two glass cuvettes. The water in one cuvette was then discarded and replaced, with the DNA samples to be analysed each in turn. This cuvette was rinsed with water and ethanol between samples.

750 μ l of diluted DNA solution were placed in the cuvette to completely fill it. Optical density measurements (A) were taken, and recorded, at 260nm and 280nm. An A₂₆₀/A₂₈₀ ratio in the vicinity of 1.8 was taken to indicate clean high molecular weight DNA. The DNA concentration was calculated by the following method:

A reading of 1 at 260nm \equiv A DNA concentration of 50 μ g/ml

A reading (R) at 260nm = A DNA concentration of (R x 50) μ g/ml

NB DNA analysed = stock DNA diluted 10 in 750 = 1:75

So, **Conc. of stock DNA (C) = (R x 50 x 75) = (R x 3750) μ g/ml**

The volume of stock containing 10 μ g of DNA was then calculated as follows:

Concentration of stock DNA (C) in μ g/ml = A₂₆₀ x 3750

Concentration of stock DNA (C) in μ g/ μ l = A₂₆₀ x 3750 x 10⁻³

Volume of stock(μ l) = 10 μ g of DNA = 10 \div (A₂₆₀ x 3750 x 10⁻³)

= 1 \div (A₂₆₀ x 0.375) μ l

= (2.67 \div A₂₆₀) μ l

CHAPTER 2

RFLP ANALYSIS

1. TISSUES

DNA extracted from paired tumour/blood samples from 20 patients was used in allele loss analysis. 10µg samples were used for each of the 40 specimens.

DNA from blood samples was taken as representative of the patients' constitutional DNA, and used as matched controls for the respective tumour DNA. In a few cases in which there was insufficient blood DNA for multiple analyses, DNA extracted from the corresponding cell line was substituted.

2. DNA DIGESTION

The volume of stock DNA containing 10µg of genomic DNA (Chapter 1, Section 2.4) was pipetted from stock vials into labelled, stoppered, Eppendorf tubes. 4µl of the buffer appropriate to the restriction enzyme to be used were added to each tube, along with a precalculated volume of sterile distilled water. The volume of distilled water was determined on the basis of a total final reaction volume of 40µl, and therefore varied with the volume of sample DNA used. 2µl (20 units) of the appropriate restriction endonuclease (Table I) were added, the reaction mixture vortexed, and briefly spun in a

microcentrifuge.

With the exception of reactions in which Taq I was used as enzyme, they were all incubated overnight, for a minimum of 16 hours, in a waterbath at 37°C. Reactions using Taq I were incubated at 65°C.

On the following day, reaction tubes were removed from the waterbath and spun briefly, to allow retrieval of droplets of condensation from the inner surface of the lid. A further 1 µl of restriction enzyme was added to each tube. The mixture was shaken and spun as previously described, and incubated at 37°C (65°C if using Taq I) for a further 3-4 hours. At the end of this incubation, the Eppendorf tubes were spun as before. 10 µl of gel loading buffer were added to each tube, vortexed and then briefly spun to bring all the contents into the bottom of the tube in readiness for loading onto an agarose gel.

TABLE I

Chromosomal locations and appropriate restriction endonucleases for probes used in RFLP analyses

CHR.	LOCUS	PROBE	ENZYME
3p	3pter-p21	pEFD 145	Rsa I
5q	5q21	pL 5.62	Bgl II
5q	5q21-22	MC 5.61	Msp I
5q	5q21	pEF 5.44	Msp I
5q	5q21-22	YN 5.48	Msp I
5q	5q21	MN 2.3	Msp I
5q	5q15-21	ECB 27	Bgl II
8q	8q22-23	TL 11	Hind III
11p	11p15.5	pEJ6.6	BamH I
11p	11pter-11p15.4	PTH	Pst I
11p	11p15.4	Calcitonin	Taq I
11p	11p13	FSH β	Hind III
13q	13q14	**p68RS 2.0	Rsa I
17p	17p13.3	**YNZ 22.1	Taq I
17p	17p13.3	**YNH 37.3	Taq I
17p	17p11.2-cen	pEW 301	Taq I
17p	17p12-13	pBHP 53	BamH I
17p	17p13	pMCT 35.1	Msp I
17p	17p13	C3068	Hae III
17q	17q23-25.3	**pTHH 59	Taq I
18q	18q21.3	pBV 15.65	Msp I
18q	18q21	SAM 1.1	EcoR I

**** = Probe detecting VNTR (Variable No. of Tandem Repeats) sequence**

3. AGAROSE GEL ELECTROPHORESIS

Digested DNA was size fractionated on 0.8% agarose gels. 3.2g of agarose (electrophoresis grade) was weighed into a conical flask, and made up to 400mls with 396.8g of 1 x TAE buffer. The total weight including a magnetic stirrer was noted, the flask covered with pierced cling film, and the agarose solution brought to the boil by heating for 3-5 mins on full power in a 650W microwave oven. The flask was reweighed and further 1 x TAE added until the original weight was obtained, to make up for any losses through evaporation. The gel solution was cooled to 65°C with constant stirring using a magnetic stirrer.

Meanwhile, a large gel electrophoresis tank was prepared (BRL Model H4). A spirit level was used in combination with the adjustable screw feet of the tank to ensure that it was level. A perspex gel tray (21x24cm) was then sealed at either end with autoclave tape, to allow a gel to be cast to a depth of about 1cm. A perspex comb with the required number of teeth (usually 22), each of approximately 60µl volume, was then inserted into comb slots provided in the tray, so that the required number of uniformly sized and spaced wells could be created when the gel was cast.

Once the gel had cooled to 65°C, it was poured into the prepared gel tank, and a pipette was used to quickly remove any trapped air bubbles. The gel was left to cool for at least 1 hour until it was completely cold and set.

The gel tray was lifted and the autoclave tape carefully peeled away from both ends of the set gel. The tray was replaced and the tank filled with sufficient 1 x TAE buffer to just

submerge the entire gel (usually 1.25-1.5l). The perspex comb was removed, taking care to leave the well bases intact, thereby ensuring that there would be no cross contamination between samples in adjacent wells once they were loaded. A small sheet of black card was placed beneath the tank to aid visualisation of the wells.

Samples were loaded from right to left, one into each well, avoiding any spillage outwith the well. For each run, a ladder marker sample (5 μ l HindIII digested lambda DNA in 25 μ l distilled water with 10 μ l gel loading buffer) was loaded into the two outermost wells, and run concurrently for band size comparison. DNA was loaded in such a way that for any given case, the tumour DNA and its corresponding constitutional DNA were loaded into adjacent wells.

When all the samples had been loaded, the lid of the gel tank was secured by attachment of the electrodes. The cathode was connected to the "well" end of the tank, and the anode to the distal end. The leads were appropriately connected to a powerpack, the current was switched on, and electrophoresis was carried out at 70V for 16-20 hours.

A pump attached to narrow bore plastic tubing was used to circulate buffer through a bucket of crushed ice. This prevented the accumulation of ions in the various compartments of the tank, and by reducing the degree of heating of circulating buffer, ensured minimal distortion of DNA tracks in the gel.

When the purple dye of the loading buffer was identified near the distal end of the gel, the current was switched off. The distal left corner of the gel was then cut to provide a marker for subsequent orientation.

4. PREPARATION OF GEL FOR SOUTHERN BLOTTING

The gel was then immersed for 30mins with gentle agitation in 1.25l of 1 x TAE buffer containing 50 μ l/l of 10mg/ml ethidium bromide. It was destained in 1.25l of distilled water for 10mins. Ultraviolet transillumination was then used to view the gel through a perspex visor, to identify any distorted tracks or any lanes with incompletely digested DNA. The transilluminated gel was then photographed. DNA in the gel was then converted to a single-stranded conformation by denaturation in 1.25l of alkaline denaturing solution with frequent gentle agitation for 45mins. The gel was then immersed in 1.25l of neutralising solution (pH 5.5) and gently agitated for 1 hour in preparation for Southern blotting.

5. SOUTHERN BLOTTING

The Southern blotting apparatus was set up using the following method. A glass plate was used to cover a rectangular plastic tray (30 x 45cm) containing 1.5l of 20xSSC. The dimensions of the glass plate (0.5 x 23 x 48cm) were such that there were gaps between the plate and the tray on each side, but not at the ends. A sheet of 17mm blotting paper with its ends immersed in the 20xSSC reservoir was used to cover the glass plate. It was thoroughly soaked with 20xSSC, and a glass pipette was gently rolled along its surface to displace trapped air bubbles. Another sheet of 3mm blotting paper cut to the dimensions of the gel was placed on top of the initial sheet, and soaked with 20xSSC.

The gel was removed from the neutralising solution, and any excess solution was drained

away. It was then placed, DNA side up, in contact with the wet paper on the blotting apparatus. A sheet of Hybond N nylon membrane (Amersham) was cut just longer than the gel; labelled with the name, date, DNA sample type, and enzyme employed; and applied to the surface of the gel. Its distal left corner was cut, as in the gel, for orientation. Trapped air bubbles were displaced as previously described, and clingfilm was wrapped around the periphery of the gel to prevent drying.

Several layers of dry absorbent materials were then placed on top of the nylon membrane, to completely cover it, in the following sequence: 2 sheets of 3mm blotting paper, cut to the same dimensions as the gel; several layers of paper towel and several thicknesses of green hand towels. A flat plate was then applied and weighted down with a 1kg brick. The apparatus was left overnight for DNA transfer.

The following day, all the layers up to the Hybond membrane were removed and discarded. The membrane was carefully peeled away from the now flattened gel, and placed DNA side (the side previously in contact with the gel) up in a UV Stratalinker (Stratagene) to covalently bind the DNA to the membrane. The membrane was then either sealed in a plastic bag, and stored at 4°C until required for hybridisation, or used immediately in hybridisation experiments. Both the gel and the blotting paper were discarded; the reservoir of 20XSSC and the glass plate were completely covered in clingfilm to reduce evaporation and stored until required for the next blotting procedure.

6. RFLP PROBES

6.1. TYPE AND SOURCE

Twenty-two restriction fragment length polymorphism (RFLP) markers were used in allele loss analysis. Twenty-one of them hybridised to RFLP sequences in the vicinity of known oncosuppressor gene loci on 6 chromosomes namely chromosomes 3, 5, 11, 13, 17 and 18. One marker, TL11, hybridised to a region which has so far not been identified as a tumour suppressor gene locus and was used for comparison as a control or "innocent" locus. A total of 8 chromosomal arms were therefore represented and these, together with the respective locus detected by each probe are detailed in Table I. Four probes, YNZ22.1, YNH37.3, pTHH59 and p68RS recognised VNTR (variable number of tandem repeats) sequences.

All probes were linearised double-stranded DNA probes. They were all obtained from colleagues involved with similar research in other malignancies at the Medical Research Council Human Genetics Unit in Edinburgh. As a result of this, all probes used had already been grown up, extracted and cleaned up, and were provided in a form ready for use in labelling reactions.

6.2. PROBE LABELLING

All probes were labelled by the hexamer multiprime method (Feinberg & Vogelstein, 1983), using the Amersham Multiprime DNA Labelling Systems Kit (RPN 1600Y).

3 μ l (25-75ng) of double stranded DNA probe were added to 17 μ l of sterile distilled water in a sterile Eppendorf tube. The probe was denatured by heating to 100°C in a

boiling water bath for 5 - 10mins. It was then 'snap' cooled on crushed ice for 2mins.

Meanwhile, other reagents were added in the following order, to another Eppendorf tube placed in an ice bath: 4 μ l of each unlabelled nucleotide - dATP, dGTP, and dTTP (nucleotides in concentrated buffer solutions containing Tris-HCl, pH 8.0, 0.5mM EDTA); 5 μ l of reaction buffer (Tris-HCl, pH 7.8, magnesium chloride and 2-mercaptoethanol), and 5 μ l of primer solution (random hexanucleotides in an aqueous solution containing nuclease-free BSA). These reagents were mixed, spun, and transferred to the tube containing the denatured probe.

Using strict precautions for the handling of radioactive isotopes, 5 μ l (50 μ Ci) of 32 P-dCTP were added to the reagent mixture, and the reaction triggered by the subsequent addition of 3 μ l (3 units) of enzyme (1 unit/ μ l DNA polymerase I 'Klenow' fragment (cloned) in 50mM potassium phosphate, pH 6.5, 10mM 2-mercaptoethanol and 50% glycerol, kept at -20°C until required and immediately replaced after use). The reaction mixture was gently mixed and briefly centrifuged to collect the contents into the bottom of the tube. Vigorous mixing and spinning was avoided as these could result in severe loss of enzyme activity.

The tube was placed in a covered lead pot which served as a radioactive shield, and incubated in a water bath at 37°C for 2 hours.

Unincorporated label was then removed by selective precipitation of labelled DNA. The following reagents were added to the probe reaction tube: 20 μ l of sonicated denatured salmon sperm DNA; 35 μ l of 3M ammonium acetate; and 200 μ l of absolute ethanol. The

solution was mixed and then chilled in a freezer at -70°C for 30mins. The resultant suspension was spun in a microcentrifuge for 30mins, following which the radioactive supernatant which contained unincorporated nucleotides was carefully aspirated and adequate disposal performed.

The pellet was then re-dissolved in 500 μl of sonicated denatured salmon sperm DNA and an excess of sonicated human placental DNA (to reduce the amount of background non-specific hybridisation). The probe was then denatured by heating to 100°C in a boiling waterbath for 10mins. It was then 'snap' cooled in crushed ice, and used directly in blot hybridisation reactions.

7. PRE-HYBRIDISATION AND HYBRIDISATION

Pre-hybridisation and hybridisation were carried out at 65°C in a Hybaid hybridisation oven. Quick-Hyb buffer was used for both procedures. Each Hybond filter was placed between two sheets of loose nylon mesh, and immersed in 2xSSC. It was then loosely rolled, DNA side up, along with the mesh, into a cylindrical shape, and inserted into a hybridisation bottle (Hybaid). 10mls of Quick-Hyb, heated to 65°C was added to each tube, allowed to circulate and prehybridisation performed for 3-4 hours while the probe was being labelled.

Using appropriate precautions for the handling of radioactive material, the labelled probe was added to a further 10mls of Quick-Hyb buffer at 65°C , in a Falcon tube, where they were thoroughly mixed. The bottle containing the pre-hybridising filter was removed

from the oven; the spent buffer was poured away; and replaced with the 10mls of hybridisation buffer containing radioactively labelled probe. The bottle was secured and put into the hybridisation oven immediately, where continuous rotation of the bottles circulated the probe. Hybridisation occurred over the following 16-20 hours.

After hybridisation, excess unbound probe was removed by washing with high stringency wash buffer (HSB) at 65°C. The hybridisation bottle was removed from the oven, its cap was unscrewed, and the radioactive buffer carefully discarded. 150mls of HSB at 65°C was added to the bottle, and it was then replaced in the oven for the first of four sequential 15 minute washes. The wash buffer was poured away and replaced with a fresh volume after each wash. For the final wash, the nylon membrane was withdrawn from the bottle, unrolled, and placed DNA side up in a flat tray containing 300mls of HSB. It was washed for a further 15mins in a shaking incubator (Gallenkamp) at 65°C.

After washing, the membranes were dried briefly (15-30secs) between 2 sheets of blotting paper, and wrapped in clingfilm in preparation for autoradiography.

8. AUTORADIOGRAPHY

Hybond filters were wrapped in clingfilm (to prevent sticking to the film or cassette) in preparation for autoradiography. Each filter was placed DNA side up, with the cut corner in the lower left corner of a labelled light proof cassette with 2 Dupont Lightning Plus Intensifying screens. All handling of film was carried out in a photographic dark room. A sheet of Kodak XAR-5 film, with its corresponding lower left corner cut for orientation,

was inserted between the DNA side of the filter and the screen, and the cassette was tightly shut.

Autoradiography was then performed at -70°C initially for only 24 hours. The film was then removed and developed in an autoradiographic developer (RG II Fuji X-Ray films processor). This initial resultant autoradiograph, though usually faint, gave some indication as to the adequacy of the washing procedure, as well as the optimum time required before development to give strong bands with minimal background.

The cassette and clingfilm-wrapped filter were then dried free from condensation before a new Kodak XAR-5 film was inserted. Autoradiography was again performed at -70°C , this time for the predicted optimum period (usually 3-14 days), after which the resultant autoradiographs were developed as previously described, and interpreted naked eye without the use of a densitometer.

9. REPROBING HYBOND N MEMBRANE FILTERS

Once a result had been obtained, filters were stripped of bound probe to facilitate reprobing with another probe at a later date. For this purpose, filters were placed in a bath of boiling 0.1% sodium dodecyl sulphate (SDS) and allowed to boil for 5 mins. The hotplate was then switched off, and the filters and SDS allowed to cool completely. The adequacy of the stripping procedure was checked either with a Geiger counter or by autoradiography (Chapter 2, Section 8) to ensure that there were no residual bands from unstripped probe. The filters were drained of excess SDS and stored while still damp in

sealed plastic bags at 4°C until required for another hybridisation procedure.

CHAPTER 3

DNA FINGERPRINTING

1. TISSUES

DNA which had been extracted from paired tumour/blood samples from 30 patients was used with a repeat unit multipriming (RUMP) method (Ferrie et al., 1991) for the production of DNA fingerprints.

Thirteen tumour/blood pairs comprised tissue from patients with cervical carcinoma, and consisted of 13 of the 20 specimens described in Chapter 2 Section 1 in which there was sufficient DNA for analysis.

The other 17 tumour/blood pairs were derived from patients bearing other common malignancies known to show high levels of allele loss, and were used to compare these levels of loss with that in cervical carcinoma. They comprised tumour/blood pairs from 5 patients with colonic carcinoma; 5 patients with breast carcinoma; 5 patients with ovarian epithelial carcinoma; 1 patient with a bowel leiomyosarcoma; and 1 patient with a mucinous cystadenocarcinoma of the appendix.

DNA was extracted as previously described.

2. MULTI-LOCUS PROBES

Multi-locus probes 33.15 and 33.6 (Jeffreys et al., 1985a&b) were used. These hybridisation probes, which are not chromosome specific, consist of tandem repeats containing a minisatellite 'core' sequence. They can simultaneously detect a number of highly polymorphic nuclei in the human genome, to generate individual specific DNA fingerprints. Probes 33.15 and 33.6 each comprise tandem repeats of various versions of the core sequence 'GGA GGT GGG CAG GA A/G G'.

Probe 33.15 has been shown to hybridise to multiple hypervariable fragments in Hinf 1 digests of human DNA (Jeffreys et al., 1985a & b). It detects 15 resolvable hypervariable fragments per individual in the 4-20 kilobase size range. It is a 592- nucleotide PstI/AhaIII fragment containing the minisatellite (AGA GGT GGG CAG GTG G)₂₉ plus 128 nucleotides of flanking human DNA subcloned into M13mp19 DNA digested with PstI plus SmaI (Jeffreys et al., 1985b).

Probe 33.6 also detects a complex set of hypervariable regions in Hinf 1 digested human DNA. It is comprised of a shortened derivative of the 'core' - [(A GGG CTG GAG G)₃]₁₈, and hybridises to a largely novel set of hypervariable fragments when compared with probe 33.15. It can detect 11 additional fragments, in the 4-20 kilobase range, which are not detected by probe 33.15. Probe 33.6 is a 720-nucleotide HaeIII fragment containing the minisatellite plus 50 nucleotides of flanking human DNA subcloned into the SmaI site of M13mp8.

The probes were obtained in vials containing approximately 80ng of purified double-

stranded DNA insert in approximately 34µl of Tris/EDTA buffer (Cell Mark Diagnostics). They were supplied mixed with a specific hexamer primer, stored at -20°C, and thawed before use in labelling reactions.

3. DNA FINGERPRINTING TECHNIQUE

3.1. *DNA DIGESTION*

Sixty DNA digests were set up from 30 tumour/blood pairs. Each contained 5µg of DNA, 80 units of Hinf 1 enzyme, 4µl of 10x Hinf 1 buffer and sterile distilled water to a total volume of 40µl. The enzyme used was of high concentration (20-80 units/µl) in order to keep the glycerol concentration (50% of the enzyme) less than or equal to 5% of the total reaction volume. The reaction tubes were incubated in a waterbath at 37°C overnight for at least 16 hours to allow complete digestion to occur.

3.2 *AGAROSE GEL ELECTROPHORESIS AND SOUTHERN BLOTTING*

For each set of 20 samples, two 0.8% agarose gels were made up as previously described (Chapter 2 Section 3). 10µl of 4x gel loading buffer was added to each tube, mixed, and the contents brought to the bottom of the tube by centrifugation, in preparation for loading onto a gel.

A 25µl aliquot of reaction product, containing 2.5µg of Hinf 1 digested DNA, was loaded into a well in each gel, so that 2 identically loaded agarose gels were run simultaneously. As before, corresponding tumour and blood DNA were run in adjacent tracks; and similar tumour types were run concurrently on the same gel. Size

fractionation was accomplished by electrophoresis at 75V for approximately 20 hours.

Following electrophoresis the gels were prepared for blotting; the DNA was transferred to Hybond N nylon membranes by Southern blotting, and covalently bound to the membranes using the Stratalinker as previously described (Chapter 2 Sections 4 & 5). They were stored at 4°C in sealed plastic bags until required for hybridisation.

3.3. *DNA MULTI-LOCUS PROBE LABELLING*

The probes 33.15 and 33.6 were radiolabelled using $\alpha^{32}\text{P}$ -dGTP. The $\alpha^{32}\text{P}$ -dGTP (Amersham) with specific activity greater than 3000Ci/mmol was thawed and mixed. Labelling buffer was thawed, mixed, and kept on ice. The vial containing the probe was removed from its packaging and placed in a boiling waterbath for 3mins. It was then cooled on wet ice for 5mins. The vial was spun in a microcentrifuge for 2secs to bring its contents into the bottom of the tube.

Reagents were then added to the vial in the following order: 57 μl of labelling buffer, 5 μl of $\alpha^{32}\text{P}$ -dGTP, 4 μl of E.coli DNA polymerase I 'Klenow fragment' (sequencing grade 1u/ μl - Boehringer No. 997 455, kept at -20°C until used and then immediately replaced). The tube contents were mixed using a pipette, and then microcentrifuged for 2secs. The vial was incubated behind a radiation shield at room temperature for 90 mins.

3.4. *MULTI-LOCUS PROBE PURIFICATION*

After radiolabelling, the probes were purified to remove unincorporated nucleotides. The shipping buffer was poured off from the Sephadex G-50 NICK column (Pharmacia LKB Biotechnology), and the column was positioned over a waste collection vessel.

The column was equilibrated with 3 sequential additions of 3.3ml of column buffer. The labelled probe reaction mixture was transferred to the top of the column using a precision pipette and sterile disposable tips, and the mixture was allowed to run into the column. A precision pipette was then used to add 400µl of column buffer to the top of the column, and the buffer was allowed to run through to waste. The waste collection vessel was then replaced with a 1.5ml screw-capped Eppendorf vial. A further 400µl of column buffer was added to the column and the column eluate containing the purified labelled probe (monitored with a Geiger counter) was collected in the vial.

The Eppendorf vial was centrifuged for 2 seconds to bring its contents into the bottom of the vial. The labelled probe was then placed in a boiling waterbath for 5mins, chilled on wet ice for 5mins, and then used immediately for hybridisation.

3.5. PRE-HYBRIDISATION AND HYBRIDISATION

Southern blot membranes each bearing tumour/blood DNA pairs from 10 patients were immersed in 500mls of wetting solution (200mM Na₂HPO₄, pH 7.2). They were transferred, DNA side down, to 500mls of prehybridisation buffer pre-warmed to 62°C in a hybridisation chamber (plastic lidded sandwich box of dimensions slightly larger than the membranes) in an incubator with a shaking platform at 62°C for 45mins.

Meanwhile, 180mls of hybridisation buffer pre-warmed to 62°C were added to another hybridisation chamber (sandwich box). The purified, boiled, labelled probe was added and carefully mixed with the hybridisation buffer. After prehybridisation, the membranes (3 per sandwich box) were transferred, DNA side down, to the hybridisation buffer

containing the appropriate probe. Membranes were handled only with forceps and care was taken to ensure that no air bubbles were trapped between them. The box was sealed and placed in a shaking incubator (Gallenkamp) at 62°C overnight for at least 15 hours.

Unbound probe was washed off by individually transferring the membranes, DNA side down, to 500mls of wash solution at 62°C in another sandwich box. It was replaced in the shaking incubator and washed for 30mins at 62°C. This step was repeated twice; each time with fresh wash solution.

3.6. *AUTORADIOGRAPHY*

After washing, the membranes were air-dried, DNA side up, on blotting paper, and then wrapped in Saranwrap (Dow chemical Co. Ltd.) while still damp. The membranes were monitored with a Geiger counter and placed in light-tight Dupont Lightning Plus cassettes for 1-3 days against Kodak XAR-5 film at -70°C. The resultant autoradiograph was interpreted naked eye, without the use of a densitometer.

3.7. *REPROBING*

Once a result was obtained, the hybridised membranes were stripped of their DNA probes in preparation for reprobing. 500mls of stripping solution and 500mls of neutralising strip solution were each prewarmed to 45°C. The membranes to be stripped were immersed in 3xSSC to wet them.

500mls of prewarmed stripping solution was then placed in a sandwich box and the membranes transferred into the box, one at a time, DNA side down, using forceps. Care was taken to ensure that no air bubbles were trapped during the procedure, and the box

was placed in a shaking incubator (Gallenkamp) at 45°C for 30mins.

Using the same technique as described above, the membranes were then transferred into a second sandwich box containing 500mls of neutralising strip solution and placed in a shaking incubator at 45°C for 30mins. The filters were then removed from the box, rinsed with 1xSSC, wrapped in clear plastic sealing film, and stored at 4°C until required for reprobing.

CHAPTER 4

HUMAN PAPILLOMA VIRUS (HPV) ANALYSES

1. POLYMERASE CHAIN REACTION (PCR) FOR HPV

1.1. *TISSUES*

Tumour DNA extracted from 55 primary cervical carcinomas, as described in Chapter 1 Section 2.2-2.4, was used in polymerase chain reactions (PCR) for detection of human papilloma viruses. DNA was diluted in sterile distilled water first to a concentration of 1µg/µl or 500ng/µl. Aliquots of this diluted DNA were then further diluted with sterile distilled water in labelled Eppendorf tubes to a concentration of 500ng/20µl, a concentration more suitable for use in PCR reactions.

1.2. *PCR PRIMERS USED FOR HPV SEQUENCES*

Computer sequence (EMBL genetics sequence database / University of Wisconsin Genetics Computer Group Software) designed primers, specific for the E6 gene of HPV types 6, 11, 16, 18, and 33 (Arends et al., 1991) were used in PCR reactions to amplify sequences from template DNA. The primer sequences are shown below:

HPV type	Primer	Sequence
6	1	CCT GTT TCG AGG CGG CTA TCC ATA
6	2	GTA CAA TTT AGC TTT ATG AAC CGC GCC TTG GTT

11	1	TGT GTG GCC AGA CAA CTT TCC CTT
11	2	TGG TTA TTT AGA TTT ATG AAG CGT GCC TTT CCC
16	1	CTG CAA GCA ACA GTT ACT GCG ACG
16	2	CAT ACA TCG ACC GGT CCA CC
18	1	AAA CTA ACT AAC ACT GGG TTA TAC A
18	2	ATG GCA CTG GCC TCT ATA GT
33	1	AAC AGT TAA AAA ACC TTT AAA
33	2	AGT TTC TCT ACG TCG GGA CCT C

1.3. *CONTROLS*

One positive and one negative control sample were included in each PCR assay. In the case of the negative control an equal volume of distilled water was used in the reaction instead of template DNA. The positive control comprised plasmid DNA of a volume equivalent to that used in the test reactions containing 1ng of the appropriate cloned HPV DNA instead of genomic DNA.

A separate PCR assay using *Ha-ras* PCR primers 589C and 597C with each tumour DNA as template was performed as an overall positive control to confirm that the PCR system was working effectively with the test DNA.

1.4. HPV PCR REACTIONS

1.4.1. *Setting Up Reactions*

PCR primers were used to amplify sequences specific for HPV 6, 11, 16, 18 and 33 from 500ng samples of tumour DNA from the first 20 patients. Primers for types 16, 18 and 33 only were used for reactions with tumour DNA from the other 35 patients.

Standard PCR reactions were set up in 100 μ l reaction volumes using 20 μ l aliquots of tumour DNA diluted to a concentration of 500ng/20 μ l as described in Chapter 4 Section 1.1. A further 100 μ l of liquid paraffin was layered onto the reaction mixture to reduce evaporation. In an attempt to prevent contamination, PCR reagents, plasmid DNA, and PCR products were stored separately; positive displacement pipettes were used to set up reactions; and all PCR reactions were set up in a Class II hood.

Each 100 μ l reaction volume comprised 20 μ l of template DNA (500ng/20 μ l for the tumour DNA; 1ng/20 μ l for the positive control plasmid DNA), 5 μ l (0.5 μ M) of each primer, 16 μ l (0.2mM) of each dNTP, 10 μ l of 10x Taq polymerase reaction buffer (Northumbria Biologicals Ltd.), 33.5 μ l sterile distilled water and 0.5 μ l (5 units/ μ l) Taq polymerase enzyme (Northumbria Biologicals Ltd.). A "mastermix" consisting of all the reagents except the template DNA was made up, and 80 μ l volumes were aliquoted into individually labelled Eppendorfs. The liquid paraffin was then added to each tube, and finally the template DNA was carefully added to the reaction mixture beneath the oily layer.

1.4.2. *PCR Incubations*

Reactions were incubated in a Hybaid Thermal Reactor. 32-35 cycles of PCR were

initiated with a 1.5min denaturation step at 94°C and terminated with a 10min. extension step at 72°C. The intervening sequence comprised 32-35 cycles of denaturation for 1min., annealing for 2min., and extension for 3min. Denaturation was accomplished at 94°C and extension at 72°C. The annealing temperature was optimised for and therefore varied according to the HPV type. For HPV types 6 and 16 annealing was accomplished at 55°C while HPV types 11, 18 and 33 required an annealing temperature of 50°C.

1.4.3. Gel Electrophoresis & Detection by UV Transillumination

PCR products were size fractionated on 3% agarose gels containing ethidium bromide. 4.5g Nu-Sieve agarose (electrophoresis grade) (FMC) and 1.5g Sea Kem agarose (electrophoresis grade) (FMC) were weighed into a conical flask, and made up to 200 mls with 1 x TBE buffer containing 50µl/l of 10mg/ml ethidium bromide solution. The total weight including a magnetic stirrer was noted, the flask covered with pierced cling film, and the agarose solution brought to the boil by heating for 2 mins on full power in a 650W microwave oven. The flask was reweighed and further TBE containing ethidium bromide was added to make up for any losses through evaporation. The gel solution was then cooled to 65°C with stirring.

Meanwhile, a small gel electrophoresis tank was set up as described in Chapter 2 Section 3 using a smaller comb to create wells of only 25-30µl volume. Once the gel had cooled to 65°C, it was poured into the prepared gel tank, and a pipette was used to quickly remove any trapped air bubbles. The gel was left to cool for 30-60 mins until it was completely cold and set.

Once set, the comb was removed from the gel as previously described (Chapter 2 Section

3) and the gel submerged in 300-500 mls of 1 x TBE containing ethidium bromide. Samples were then loaded from right to left in the wells as previously described.

Each sample comprised 20µl of PCR product and 5µl of 4 x gel loading buffer. A 1kb lambda marker sample comprising 3.5µl of 1kb lambda ladder, 5µl of 4 x loading buffer, and 16.5µl of distilled water was loaded concomitantly into each of the two outermost wells of the gel for band size comparison. Electrophoresis was accomplished at 75-100V over approximately 90 mins.

Following electrophoresis, the presence or absence of appropriately sized product bands was detected by ultraviolet transillumination of the gel. The transilluminated gel was then photographed.

2. INTEGRATED VERSUS EPISOMAL STATUS OF HPV

2.1. *TISSUES*

The status of the human papilloma virus DNA with respect to integration was determined for 15 of the HPV positive tumours. These tumours were amongst the first 20 tumours analysed in which there was sufficient tumour DNA available for this analysis. In the latter 35 cases, there was insufficient material for this type of analysis.

2.2. *DNA DIGESTION, GEL ELECTROPHORESIS AND SOUTHERN BLOTTING*

10µg samples of tumour DNA were digested with either BamHI (HPV 16 positive

cases), ECoRI (HPV 18 positive cases), or BglII (HPV33 positive case). Reactions were set up in 40µl volumes comprising 10µl (1µg/µl) tumour DNA, 4µl of the appropriate restriction enzyme, 4µl of its corresponding 10 x buffer, and 12µl sterile distilled water, incubated for 16-20hrs overnight at 37°C prior to the addition of 10µl of 4 x gel loading buffer.

The digested samples were then electrophoresed on 0.8% agarose gels, and Southern blotted onto Hybond N+ membranes in preparation for DNA hybridisation. The methods used were as previously described in Chapter 2 Sections 3-5 except that because of the positive charge on Hybond N+ membranes the following alkaline blotting procedure was used.

After denaturing for 45mins as previously described, a Southern blot was set up using denaturing solution rather than 20 x SSC. Further, instead of using the Stratalinker to bind DNA covalently to the filter, the filter was washed in neutralising buffer for 40mins after the blotting procedure. It was then dried between two sheets of blotting paper and stored as previously described, or used directly for hybridisation reactions.

2.3. LINEARISATION & RADIOLABELLING OF PLASMID HPV DNA PROBE

HPV 16, 18 and 33 plasmid DNA were each linearised with the appropriate restriction endonuclease - BamHI, ECoRI or BglII, respectively. Reactions were set up in 20µl volumes comprising 2µg (1µg/µl) plasmid DNA, 2µl of the appropriate restriction enzyme, 2µl of that enzyme's 10 x buffer, and 14µl of sterile distilled water. The reactions were incubated in a waterbath at 37°C for 16-20hrs overnight.

The linearised HPV DNA (2µg/20µl) was diluted 1:2 with sterile distilled water to a concentration of 50ng/µl, and labelled for use as a probe in hybridisation reactions with the Southern blots of tumour DNA.

For each probe 50ng (1µl) of diluted linearised plasmid HPV DNA was mixed with 28µl of sterile distilled water in an Eppendorf tube. The DNA was then denatured by boiling for 5mins in a waterbath, and 'snap' cooled on ice for a further 2mins. 10µl hexanucleotide mix (Amersham), 5µl primer (Amersham), and 4µl ³²P-dCTP were then added to the tube in that order, and the labelling reaction triggered by the addition of 2µl DNA polymerase (Klenow fragment) (Amersham). The 50µl labelling reaction mixture was shielded in a lead vial and incubated in a waterbath at 37°C for 30 mins.

The reaction mixture was then filtered through a Sephadex Nick Column (Pharmacia) to remove unincorporated nucleotides. The shipping buffer was poured off the column, and 400µl of TE buffer was added and allowed to drip through to waste. A further 400µl of TE buffer was mixed with the 50µl probe and the resultant mixture loaded onto the column and allowed to drip through to waste. A further 400µl of TE buffer was then loaded onto the top of the column and the eluate consisting of the labelled probe collected into an Eppendorf tube. This was stored at -20°C in a lead pot until its use in hybridisation reactions.

2.4. HYBRIDISATION, WASHING AND AUTORADIOGRAPHY

Filters were soaked in 2 x SSC, and loosely rolled into hybridisation bottles as previously described (Chapter 2 Section 7). As positively charged Hybond N+ membranes were used, Hybridisation Solution rather than Quick Hyb was used as the hybridisation buffer.

500µl of salmon sperm DNA (10mg/ml) was denatured by boiling for 5mins, 'snap' cooled on ice, mixed with 25ml of Hybridisation Solution and added to the hybridisation bottles which were then rotated in a hybridisation oven (Hybaid) for 3-4 hours at 65°C to effect prehybridisation.

Once prehybridisation was complete, the labelled probe was denatured by boiling for 5 mins, added to an aliquot of prewarmed hybridisation solution from the contents of the prehybridising bottle, and then replaced in the bottle and circulated amongst the filters. Hybridisation was performed for 16-20hrs overnight at 65°C.

Post-hybridisation washes were also carried out at 65°C and consisted of 2 x 10min washes in 2 x SSC, 3 x 10min washes in 2 x SSC / 1% SDS and 3 x 10min washes in 0.5 x SSC / 1% SDS in that order. 150mls of wash buffer was used in each bottle.

Filters were then air dried, and autoradiography was performed for 1-5 days as previously described (Chapter 2 Section 8). Band sizes on the resultant autoradiographs were compared with the 6- 8kb size of the linearised HPV DNA fragment. The status of the virus in the tumour samples was interpreted as episomal if the size of the tumour HPV band was the same as that of the linearised HPV DNA; and likely to be integrated if the size/s of the band/s varied from that of linearised HPV DNA.

CHAPTER 5

DETECTION OF p53 MUTATIONS BY POLYMERASE CHAIN REACTION (PCR) FOLLOWED BY DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE)

1. TISSUES

Cervical tumour DNA, described in Chapter 1, from 55 patients, diluted to a concentration of 500ng/20 μ l (see Chapter 4 Section 1.1) was used as template in these analyses. The corresponding blood DNA from the first 20 patients was also amplified in the PCR reactions. However, for the latter 35 cases, it was only for those in which a putative mutation was detected in the tumour DNA, that it was deemed necessary to compare the constitutional profile, by electrophoresis of the blood products on denaturing gradient gels.

2. p53 PCR PRIMERS

Five pairs of PCR primers were synthesised on a DNA synthesiser (Applied Biosystems) and used in PCR reactions. A "GC-clamp", previously described by Borresen (Borresen et al., 1991) was attached to one primer of each pair, creating a 60-mer primer. The other primers were all 20-mers.

With the exception of one pair of primers (for convenience designated the "E" primers)

each pair of primers amplified across 1 of the 4 "hot-spot" regions (HSR), A-D of the p53 gene. The non-primer regions of the amplified fragments corresponded to exon 5's codons 128-153 for HSR A; exon 5's codons 155-185 for HSR B; exon 7's codons 237-253 for HSR C; and exon 8's codons 265-301 for HSR D of the p53 gene (Borresen et al., 1991). The non-primer region of the fragment amplified by the "E" primers corresponded to exon 6's codons 193-218.

The following primer pairs were constructed :

Fragment A :

Sense primer - (5'-TTCCTCTTCCTGCAGTACTC-3');
 Antisense primer - (5'-CGGCACCCGCGTCCGCGCCACGGGCGGGGGCG
 GCGGGACGGGCGCGGGGCGCGGCGGGCG-3')

Fragment B :

Sense primer - (5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCC
 CCGCCCGTTCCACACCCCCGCCCGGCA-3');
 Antisense primer - (5'-GATGGTGAGCAGCTGGGGC-3')

Fragment C :

Sense primer - (5'-CACCATCCACTACAACACTACA-3');
 Antisense primer - (5'-CATCATCACACTGGAAGACTGCCCGCCCCCGC
 CGCCCTGCCCGCGCCCCGCGCCGCCCCGC-3')

Fragment D :

Sense primer - (5'-ATCCTGAGTAGTGGTAATCT-3');
 Antisense primer - (5'-AGGGAGCACTAAGCGAGGTAGCCCGCCCCCG
 CCGCCCTGCCCGCGCCCCGCGCCGCCCCGC-3')

Fragment "E"

Sense primer - (5'-GTCTGGCCCCCTCCTCAG plus GC-clamp-3')

Antisense primer - (5'-CCCTATGAGCCGCCTGAG-3')

The synthesised primers were provided dissolved in ammonia in 20µl volumes. 20µl of 3M sodium acetate (pH 5.5) were added to each primer, following which each Eppendorf was filled with absolute ethanol. The tube was gently agitated, and incubated at -20°C for 30mins. After centrifugation (3000 rpm) for 5mins, the supernatant was decanted off, and the pellet was washed in 2- 3 changes of 80% ethanol. The residual pellet was then air dried for 15 mins, and dissolved in 100µl of TE buffer by gentle agitation with the pipette.

The stock primer concentrations were then calculated from the optical density measurements at a wavelength of 260nm on the spectrophotometer as previously described for genomic DNA (Chapter 1, Section 2.4). Stock primers were then diluted in sterile distilled water to a concentration of 100pmol/5µl in readiness for use in PCR reactions.

3. p53 PCR TECHNIQUE

The method used was similar to that described for HPV (Chapter 4 Section 1.4.1). 500ng samples of tumour or blood DNA (500ng/20µl) were used as template, with 100pmol (100pmol/5µl) of each of a pair of matched primers, 0.2mM of each dNTP (16µl of a solution comprising 10µl each of dATP, dCTP, dGTP and dTTP (NBL) in 760µl doubly

distilled water), 10 μ l of Taq polymerase 10x buffer (NBL), 2.5 units (5 units/ μ l) Taq polymerase enzyme (NBL), and 43.5 μ l doubly distilled water, to a total volume of 100 μ l per reaction.

As described in Chapter 4 Section 1.4.1, a further 100 μ l of liquid paraffin was added to each reaction tube to reduce evaporation. Reactions were set up as previously described with an initial "mastermix", and taking suitable precautions; and the PCR incubations were conducted in a Hybaid Thermal Cycler.

Thirty-five cycles of denaturing for 45secs at 94°C, annealing for 45secs at 55°C, and extension for 45secs at 72°C, were initiated with a 7min denaturation at 94°C, and ended with a 10min extension at 72°C (Borresen et al., 1991).

4. GEL ELECTROPHORESIS & DETECTION BY UV TRANSILLUMINATION

As previously described for HPV (Chapter 4 Section 1.4.3), the presence of amplified product was confirmed by electrophoresis in a 3% agarose gel followed by UV transillumination and the gels were photographed. Any unsuccessful reactions were repeated (DNA permitting) until products were obtained. All amplified products were stored at -20°C.

5. DENATURING GRADIENT GEL ELECTROPHORESIS OF PCR PRODUCTS

Amplified products were screened for the presence of p53 mutations by parallel

denaturing gradient gel electrophoresis (DGGE) (Sheffield et al., 1989; Borresen et al., 1991). 50µl samples of amplified product from PCR reactions (Chapter 5 Section 3) involving 5 pairs of primers for 5 regions of the p53 gene (Chapter 5 Section 2) in 47 cervical tumours were aliquoted for use in DGGE.

For each region, a positive control consisting of DNA from another tumour known from previous sequencing experiments to possess a mutation in that region was run concomitantly with the cervical tumour DNA samples. Likewise, DNA from a specimen with unmutated or wild-type sequence for that region was also run with the test specimens for comparison.

Denaturing gradient polyacrylamide gels were cast using a 2- channel pump with a mixing chamber to simulate a gravitational gradient mixer. Reactions with the A primers were run on a 50-80% gel, while those with primers B, C, D and "E" were run on 35-80% denaturing gels. All reagents used were of electrophoretic grade.

30µl aliquots of gel loading buffer, bromophenol blue, xylene cyanol and PCR product (each sample being the result of amplification of DNA from 1 tumour with 1 pair of primers) were each loaded into a well (usually 15 wells per gel) along the top of the gel. Gels were run submerged in 1 x TAE buffer in a standard Protean electrophoresis apparatus (BioRad) contained in a water bath at 55°C and at 400V. The electrophoresis direction was parallel to that of the denaturing gradient, and the glass plates surrounding the gels were in direct contact with the buffer on both sides.

Electrophoresis was deemed complete when the xylene cyanol blue had just run off the

bottom end of the gel. The gels were stained with ethidium bromide (0.5µg/ml) in 1xTAE buffer for 5 mins, viewed by UV transillumination and photographed.

6. ANALYSIS OF TUMOUR DNA BY SEQUENCING OF p53 GENE REGIONS

Amplified products from both tumour and the corresponding constitutional (blood) DNA of any samples identified as having a DGGE mobility that varied from that of the wild type sequence of that region of p53 were sequenced. DNA from 3 cases was sequenced, and the following method was employed.

Each band to be sequenced was cut out of the gel and passively eluted for a minimum of 24 hours into 200µl of TE buffer. 2µl of the eluate was used in 100µl of a new PCR reaction, the product of which was purified with Gene Clean (BIO 101, California). Sequencing was carried out by the method of Winship (1989), using the Sequenase 2.0 kit (U.S.B., USA) with ³⁵S-dATP.

CHAPTER 6

DNA PLOIDY ANALYSIS BY FLOW CYTOMETRY

1. TISSUES

Samples of fresh tumour tissue from 52 patients with cervical carcinoma, obtained as described in Chapter 1 Section 1, and stored at -70°C were used for ploidy analysis. Samples were analysed in groups of ten. In the majority of cases, tumour specimens were small, and, because of the limited availability of samples, only one sample from each tumour could be used for ploidy analysis. In most cases, the ploidy result was therefore derived from analysis of a single random sample.

2. PREPARATION OF SAMPLES FOR ANALYSIS

The Vindelov method of analysis (Vindelov et al., 1983) was used. Each sample, of approximately $1\text{-}2\text{mm}^3$ dimension, was removed from the freezer immediately prior to use, and thawed just sufficiently to allow cutting with a scalpel blade.

The tumour sample was finely minced and suspended in $0.5\text{-}1.0\text{ml}$ of citrate buffer. This suspension was gently agitated with a pipette, and a $100\mu\text{l}$ aliquot was transferred to a perspex tube.

$3\mu\text{l}$ of reference standard chicken red cells were then added to the tube and mixed. $450\mu\text{l}$

of Solution A were then added to trypsinise the cells; the solution was mixed by vortexing, and incubated for 10min at room temperature.

325µl of Solution B containing trypsin inhibitor and ribonuclease A were then added to the reaction mixture. The resultant solution was again agitated by vortexing, and incubated for a further 10min at room temperature.

Solution C containing the propidium iodide used for staining the nuclei was kept on ice and protected from light at all times. 250µl of this solution were added to each reaction tube on ice. The reagents were mixed by vortexing, and incubated on ice for a further 10min.

In an attempt to remove any solid particles or debris, the resultant 1.125ml reaction mixture was filtered prior to analysis in the flow cytometer, by pipetting it into the barrel of a 5ml syringe in which a small thickness of gauze swab had previously been placed. It was then filtered into a clean perspex tube through a 21-gauge hypodermic needle, and kept on ice awaiting analysis.

3. SAMPLE ANALYSIS

An EPICS-C flow cytometer was used for sample analysis. The samples were fed through the flow cytometer, and analysed using laser light at an excitation wavelength of 488nm. The resultant light scatter was analysed to give an indication of cell size, granularity and resultant red fluorescence. The red fluorescence of the propidium iodide stained cells

detected by the flow cytometer is stoichiometric with the cellular DNA content.

Samples were kept on ice until analysed. The probe of the sampling channel was flushed with distilled water prior to analysis of each sample and then sheathed. The sample to be analysed was agitated by vortexing, and the perspex tube inserted into the sampling chamber. The sample was then fed through the flow cytometer, a display was obtained on the VDU screen, and the sampling device was sheathed.

For each sample approximately 10,000 cell nuclei were analysed and the resultant DNA histogram displayed on the VDU screen was scaled. The mean DNA content values (G_0/G_1 and G_2M) of the standard reference and tumour sample cell populations were recorded, and the coefficients of variation automatically calculated and recorded on screen. The entire graphical illustration was printed out as well as saved onto computer disk.

4. INTERPRETATION OF TUMOUR PLOIDY FROM DNA HISTOGRAMS

The ploidy status of 52 tumours was interpreted from the resultant histogram of flow cytometric analysis of a single sample from each tumour according to the Convention on Nomenclature for DNA Cytometry (Hiddemann et al., 1984).

The DNA index is the ratio of the mode (or mean) of the relative DNA content of the G_0/G_1 cells of the sample divided by the mode (or mean) of the relative DNA measurement of the diploid G_0/G_1 reference cells. Cells with a normal diploid karyotype

have, by definition, a DNA index of 1.0 (Hiddemann et al., 1984). The DNA index (DI) of the chicken red cells was taken to be 0.36.

A population of cells with a DI of 1.8-2.2, and in excess of normal ratios was interpreted as tetraploid. For the purposes of this study, these populations, together with populations of cells in which $1 < DI < 1.8$ and for which a corresponding G_2M peak could be identified, were classified as aneuploid.

Samples with two clear peaks - G_0/G_1 (DI 0.9-1.1) and G_2M (DI 1.8-2.2) with normal ratios - in the absence of any aneuploid populations were classified as diploid.

CHAPTER 7

IMMUNOHISTOCHEMISTRY

1. p53 IMMUNOHISTOCHEMISTRY

1.1. *TISSUES*

Cervical biopsies from 115 patients were obtained from several sources. They included routine archival punch biopsies from patients who had attended for colposcopy; fresh tissue from patients undergoing Wertheim's hysterectomy or radiotherapy for cervical carcinoma (Chapter 1 Section 1); and specimens from patients without history or evidence of cervical disease, undergoing simple hysterectomy for benign uterine conditions. A spectrum of pathological changes from normal mucosa through preinvasive lesions to invasive carcinoma (Table II) was represented in the specimens examined.

TABLE II

p53 Immunohistochemistry: Histological Appearances in 115 Cervical Biopsies

HISTOLOGY	NO. OF CASES
Squamous carcinoma	51
Adenocarcinoma	3
Adenosquamous carcinoma	3
CIN3	46
CIN 1 or 2	24
Koilocytosis	22
Glandular atypia / adenocarcinoma-in-situ	3
Benign metaplastic changes in glands	9
Normal squamous epithelium	53
Normal endocervical glands	62

In the majority of cases, tissue samples had been fixed immediately in formalin or freshly prepared periodate-lysine-paraformaldehyde-dichromate (PLPD) (Pollard et al., 1987). However in a few cases samples had been 'snap' frozen in liquid nitrogen prior to fixation in PLPD. Specimens fixed in formalin were processed in routine fashion to paraffin. Those fixed in PLPD were fixed over 24hrs, rinsed in running water over 24hrs to remove the dichromate pigment, processed by a method that completely avoided formalin, and embedded in paraffin.

1.2. p53 ANTIBODIES

Two human p53-specific mouse monoclonal antibodies, PAb 1801 (Cambridge Bioscience) and MAb DO-7 (a gift from Prof. David Lane, CRC Laboratories, Dundee), were used in these analyses. PAb 1801 and MAb DO-7 bind to different epitopes on the p53 oncoprotein. Both bind to the linear fragment of human p53 consisting of the N-terminal 91 amino acids (AA), but they are sterically distinct, and the epitope recognised by PAb 1801 probably lies between AA 1-91, while that recognised by MAb DO-7 lies between AA 37-45. PAb 1801 does not recognise the 1-45 construct (Vojtesek et al., 1992).

PAb 1801 was used only on PLPD-fixed material as the epitope which it recognises is destroyed by formalin fixation. MAb DO-7 was used on both PLPD and routinely formalin-fixed tissue. Formalin and PLPD-fixed specimens of a colonic carcinoma, known to express mutant p53 strongly, were used as positive controls.

Previous work in the laboratory with PAb 1801 on colonic carcinomas had suggested a 90min incubation, and an optimal working dilution of 1:100 for this antibody. For

antibody MAb DO- 7 titration experiments were carried out on PLPD- and formalin-fixed positive control sections with serial dilutions of 1:50, 1:100, 1:250, 1:500 and 1:1000 in 2hr versus overnight incubations. Optimal conditions of a 1:250 dilution incubated at 4°C overnight were used for formalin-fixed tissues; while a 1:1000 dilution of antibody incubated for 2 hours was used for PLPD-fixed tissues.

1.3. *p53 IMMUNOHISTOCHEMICAL TECHNIQUE*

An avidin-biotin complex (ABC) immunohistochemical method for monoclonal antibodies was employed. 4µm sections of paraffin embedded tissue were cut, floated onto poly-L-lysine-coated glass slides, dried at low temperature, and stained using monoclonal antibodies, PAb 1801 and MAb DO-7. Approximately 20 sections were stained in each run. Positive and negative (primary antibody withheld) controls were included in each run.

Sections were deparaffinised in xylene for 5mins, and rehydrated for 30secs in graded (100% → 74%→ 64%) alcohols to water. Sections were then washed in water for 5mins prior to a further 5min wash in either Tris buffered saline (TBS) or phosphate buffered saline (PBS). In accordance with the manufacturer's instructions, TBS was used throughout incubations with PAb 1801; while PBS was used in those involving MAb DO-7. Prior to incubation with the primary antibody, sections were incubated for 10min with normal rabbit serum diluted 1:5 in TBS (NRS/TBS) or PBS (NRS/PBS).

Sections fixed in PLPD were then exposed to the primary antibody diluted 1:100 in TBS (PAb 1801) or 1:1000 in PBS with 10% fetal calf serum (10% FCS) (MAb DO-7) for 2hrs. Formalin-fixed sections were incubated at 4°C overnight with the primary

antibody, MAb DO-7 diluted 1:250 in PBS /10% FCS.

Biotinylated rabbit anti-mouse immunoglobulin (DAKO No. E354) incubated for 2hrs at a dilution of 1:50 in NRS/TBS or NRS/PBS was used as the secondary antibody on all sections. Horseradish- peroxidase linked avidin-biotin complex (DAKO ABComplex No. K355) made up at least 30 mins prior to use was used in the final 30min incubation stage. Two x 5min washes with either TBS or PBS were performed between all incubations.

3'3-diaminobenzidine (Sigma) at a concentration of 1mg/ml in the presence of 0.03% hydrogen peroxide was used for visualisation. The final visualisation reaction (development of a brown precipitate) was stopped after 2-3min by rinsing in running cold water. Sections were then lightly counterstained with haematoxylin, washed in water, destained in Scott's tap water substitute, rewashed in water, dehydrated in graded (64% → 74% → 100%) alcohols, cleared in xylene and mounted with DPX and glass coverslips.

Stained sections were viewed by light microscopy, and all results were formally checked by a gynaecological pathologist.

2. *ras* p21 IMMUNOHISTOCHEMISTRY

2.1. *TISSUES*

Eighty-nine cervical biopsies were obtained as described in Chapter 7 Section 1.1. Fewer cases were examined for *ras* than for p53 as only material which had been fixed in PLPD

could be used in *ras* analyses. The histological appearances in these 89 cervical biopsies are tabulated in Table X of the Results. Sections taken from a block of a PLPD-fixed breast carcinoma, known from previous experiments (Going, 1989) to express *ras* strongly were used as positive controls.

2.2. *ras* p21 ANTIBODY

A rat monoclonal antibody, Y13 259 (Furth et al., 1982), which recognises the 21kD *ras* protein (p21) was used in these analyses. This antibody, which was produced from the hybridoma by Mr. R. Morris in the CRC Laboratories at Edinburgh University Medical School Pathology Department, was raised against a *ras*-expressing cell line and detects the epitope on either PLPD-fixed or frozen material. It recognises the protein products of *N-ras*, *Ha-ras*, and *Ki-ras*, in both wild type and mutated forms.

Titration experiments, using serial dilutions (1:50, 1:100, 1:1000) of primary antibody on the positive control tissue, revealed an optimal staining result (strongly positive staining with minimal background) with the 1:50 dilution.

2.3. *ras* IMMUNOHISTOCHEMICAL TECHNIQUE

An ABC method (previously described in Chapter 7 Section 1.3) was used. Positive and negative (primary antibody omitted) controls were included with each run. Tris buffered saline (TBS) was used in all incubations with Y13 259; and, with a few exceptions detailed below, the procedure used was the same as that described for PAb 1801 (Chapter 7 Section 1.3).

Normal goat serum (NGS) was used instead of normal rabbit serum; the primary antibody

Y13 259 was diluted 1:50 in a 1:5 solution of NGS/TBS; and a 1:50 dilution of goat anti-rat immunoglobulin in 1:5 NGS/TBS was used as secondary antibody.

PART III

RESULTS

PART III RESULTS

CHAPTER 1 CASE DISTRIBUTION

CHAPTER 2 ALLELE LOSS ANALYSIS

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CHAPTER 4 HPV STATUS OF TUMOURS

1. HPV status of tumours examined for allele loss
2. HPV analysis of all cervical tumours examined

CHAPTER 5 p53 ANALYSIS

1. Screening for p53 mutations by PCR followed by DGGE
2. p53 immunohistochemical analysis
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CHAPTER 6 ras p21 IMMUNOHISTOCHEMICAL ANALYSIS

1. Case distribution
2. ras p21 immunohistochemical staining of control tissues
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CHAPTER 7 DNA PLOIDY ANALYSIS BY FLOW CYTOMETRY

1. Incidence of DNA aneuploidy
2. DNA ploidy and clinicopathological parameters

CHAPTER 1

CASE DISTRIBUTION

A total of 55 fresh cervical carcinoma specimens were collected. Their distribution by patient age, histological type, FIGO stage and degree of tumour differentiation is shown in Table III. Tumours have been listed in the order in which they were collected and their assigned numbers 1 to 55 are shown. Patient ages ranged from 23 to 72 years, with a median age of 47 years. 11 specimens were obtained from patients under 35 years of age, 23 were between 35 and 55 years, and 21 fell into the over 55 years category.

There were 49 squamous carcinomas, 3 adenocarcinomas and 3 adenosquamous carcinomas. At diagnosis, 24 carcinomas were at stage I, 22 stage II, 7 stage III and 2 stage IV. Review of the histology reports on these tumours revealed that the degree of differentiation had been reported in 31 cases, of which 2 had been classified as well differentiated, 15 as moderately differentiated and 14 as poorly differentiated.

An unfortunate laboratory mishap (freezer breakdown that went unnoticed for several days) resulted in several samples from tumour specimens perishing beyond redemption, and severely limited the DNA available for analysis in the latter 35 cases (nos. 21 - 55). This dictated that analyses on tumours 21-55 were limited to those, like PCR, which required relatively small quantities of DNA. A complete set of results with respect to the presence of HPV types 16, 18 and 33; mutational status of p53 as determined immunohistochemically, and by denaturing gradient gel electrophoresis of the PCR

amplified products of the hot spot regions; and DNA ploidy status analysed by flow cytometry, is therefore available for 45 cervical carcinomas. Amongst these are 20 for which allele loss analysis has been performed, and for which the status (integrated vs. episomal) of the HPV, where present, has been ascertained. The DNA fingerprints of 13 of these tumour/blood pairs have been examined. Of the remaining 10 tumours in which complete data sets are not available, there was sufficient tumour/DNA sample to perform some but not all of the analyses.

Results have also been obtained from immunocytochemical analysis of ras and p53 expression in a large series (89 and 115 cases respectively) of cervical biopsies showing normal, pre-invasive and invasive cancer histology.

TABLE III Clinicopathological features of 55 cervical carcinomas studied

CASE NO	AGE	HISTOLOGICAL TYPE	GRADE	FIGO STAGE
1	58	ADENOCARCINOMA	P	IIB
2	31	SQUAMOUS CARCINOMA	M/P	IB
3	23	ADENOSQUAMOUS CARCINOMA	NR	IB
4	43	ADENOCARCINOMA	W	IB
5	67	SQUAMOUS CARCINOMA	M	IIIB
6	41	SQUAMOUS CARCINOMA	NR	IB
7	37	SQUAMOUS CARCINOMA	P	IB
8	30	SQUAMOUS CARCINOMA	M	IB
9	34	SQUAMOUS CARCINOMA	P	IIB
10	36	ADENOCARCINOMA	NR	IB
11	32	SQUAMOUS CARCINOMA	M	IVA
12	69	SQUAMOUS CARCINOMA	P	IIB
13	62	SQUAMOUS CARCINOMA	M	IIB
14	25	SQUAMOUS CARCINOMA	P	IB
15	61	SQUAMOUS CARCINOMA	NR	IB
16	41	SQUAMOUS CARCINOMA	M	IB
17	41	SQUAMOUS CARCINOMA	NR	IB
18	70	SQUAMOUS CARCINOMA	P	IIB
19	54	SQUAMOUS CARCINOMA	W	IIB
20	67	SQUAMOUS CARCINOMA	NR	IIIB
21	50	ADENOSQUAMOUS CARCINOMA	P	IIB
22	36	SQUAMOUS CARCINOMA	NR	IB
23	39	SQUAMOUS CARCINOMA	NR	IIA
24	63	SQUAMOUS CARCINOMA	NR	IB
25	69	SQUAMOUS CARCINOMA	NR	IIA
26	57	SQUAMOUS CARCINOMA	M	IB
27	47	SQUAMOUS CARCINOMA	NR	IIIB
28	43	SQUAMOUS CARCINOMA	NR	IIB
29	69	SQUAMOUS CARCINOMA	NR	IIB
30	61	SQUAMOUS CARCINOMA	NR	IIB

TABLE III continued

CASE NO	AGE	HISTOLOGICAL TYPE	GRADE	FIGO STAGE
31	40	SQUAMOUS CARCINOMA	NR	IB
32	31	SQUAMOUS CARCINOMA	NR	IB
33	25	SQUAMOUS CARCINOMA	NR	IB
34	62	SQUAMOUS CARCINOMA	NR	IB
35	61	SQUAMOUS CARCINOMA	NR	IIB
36	57	SQUAMOUS CARCINOMA	P	IIIA
37	60	SQUAMOUS CARCINOMA	M	IIA
38	53	SQUAMOUS CARCINOMA	P	IIIB
39	63	SQUAMOUS CARCINOMA	M	IIIB
40	43	SQUAMOUS CARCINOMA	NR	IB
41	34	SQUAMOUS CARCINOMA	M	IB
42	72	SQUAMOUS CARCINOMA	NR	IIB
43	52	SQUAMOUS CARCINOMA	M/W	IIA
44	30	SQUAMOUS CARCINOMA	P	IB
45	32	SQUAMOUS CARCINOMA	P	IB
46	69	SQUAMOUS CARCINOMA	M	IIB
47	46	SQUAMOUS CARCINOMA	M/P	IIB
48	47	SQUAMOUS CARCINOMA	NR	IIA
49	40	SQUAMOUS CARCINOMA	P	IB
50	43	SQUAMOUS CARCINOMA	P	IIB
51	51	ADENOSQUAMOUS CARCINOMA	P	IIB
52	66	SQUAMOUS CARCINOMA	M/P	IIB
53	54	SQUAMOUS CARCINOMA	NR	IVA
54	42	SQUAMOUS CARCINOMA	M	IB
55	67	SQUAMOUS CARCINOMA	NR	IIA

P = poorly differentiated; M = moderately differentiated; W = well differentiated; NR = not recorded

CHAPTER 2

ALLELE LOSS ANALYSIS

Twenty tumour-blood pairs from patients with cervical carcinoma were analysed. For any given polymorphic probe, the presence of two distinct bands (alleles) on the resultant autoradiograph of the constitutional DNA was interpreted as heterozygosity for that individual at the locus defined by that probe; and the case was regarded as informative. Loss of one band (allele), or a significantly diminished intensity of signal, not reflected in the remaining band (allele), in the corresponding tumour DNA was regarded as loss of heterozygosity. Cases showing only one band in both constitutional and tumour DNA were taken to be homozygous for that particular locus, and, as no inferences could be drawn from these results, they were regarded as uninformative (Fig. 3).

The use of 22 polymorphic DNA probes on paired tumour/blood DNA from 20 patients with cervical carcinoma generated 211 analyses showing constitutional heterozygosity. The results of these 211 analyses in terms of the frequency of loss of heterozygosity (LOH) observed with each polymorphic probe are given in Table IV. For each of the 22 marker loci on 8 autosomal chromosomal arms, at least 2 and up to 15 cases were informative. In general, a low overall incidence of LOH was reflected at each oncosuppressor site tested.

Fifteen of the 22 markers revealed LOH in one or more of the informative cases. The frequency of LOH amongst informative cases (Table IV) ranged from 7% (1 LOH in 15 informative cases with probe pEJ6.6 and 1 LOH in 14 informative cases with PTH) to

30% (3 losses seen amongst 10 informative cases with Calcitonin). An incidence of 50% LOH occurred with only one probe, pL5.62, in which 1 of only 2 informative cases showed LOH. However, over the whole series, of 211 informative loci, only 22 sites (10%) distributed amongst 9 tumours showed LOH.

The majority (11) of the 20 tumours showed no losses at any tested site, and 12 of the examples of LOH were found in just 2 of the 20 tumours (nos. 13 and 16). Clinically and histologically, these two tumours (both squamous carcinomas) did not appear to differ from the others. Both tumours were moderately differentiated. Tumour 13, a stage IIb carcinoma, was from a 62 year old patient; while tumour 16 was a stage Ib cancer from a 41 year old.

Addressing specific chromosomal sites in turn:

Chromosome 17

The combined result with 6 RFLP markers on chromosome 17p detected LOH at one or more loci in only 3 of 20 informative cases. All the LOH observed was compatible with deletion involving the p53 gene in the vicinity of 17p.13. No losses were observed amongst 13 informative cases on chromosome 17q using the VNTR probe THH59.

Chromosome 13

No losses were identified amongst 14 informative cases at the locus defined by p68RS2.0, within the Rb gene on chromosome 13q.

Chromosome 11

On chromosome 11p, the calcitonin locus, the c-Ha-ras locus defined by marker pEJ6.6,

and those defined by markers PTH, and FSHB were analysed. Five losses occurred amongst 19 informative cases. Three cases exhibited LOH at the calcitonin locus, one loss was observed at loci defined by each of the markers pEJ6.6 and PTH, and no losses were seen in four cases informative with FSHB.

Chromosome 5

The combined result of 6 RFLP markers pL5.62, MC5.61, pEF5.44, YN5.48, MN2.3, and ECB 27 in the 5q.21 region on chromosome 5, revealed LOH in 5 of 16 informative cases. Losses were detected with pL5.62, pEF5.44, and YN5.48, while none was observed with MC5.61, MN2.3 or ECB 27.

Chromosome 3

Only 1 loss was observed amongst 4 informative cases using the probe pEFD 145 which recognises a sequence within the chromosome 3p.21 band.

Chromosome 18

Two markers were used to detect losses on the long arm of chromosome 18 - pBV15.65 and SAM 1.1. Of 12 informative cases, 3 showed losses - 2 cases at the locus defined by SAM 1.1, and the other at both loci.

Chromosome 8

Lastly, one loss was observed in 7 informative cases (14%) at the locus defined by marker TL11 on chromosome 8q - so far not implicated as an oncosuppressor site, and hence useful as a "control" site.

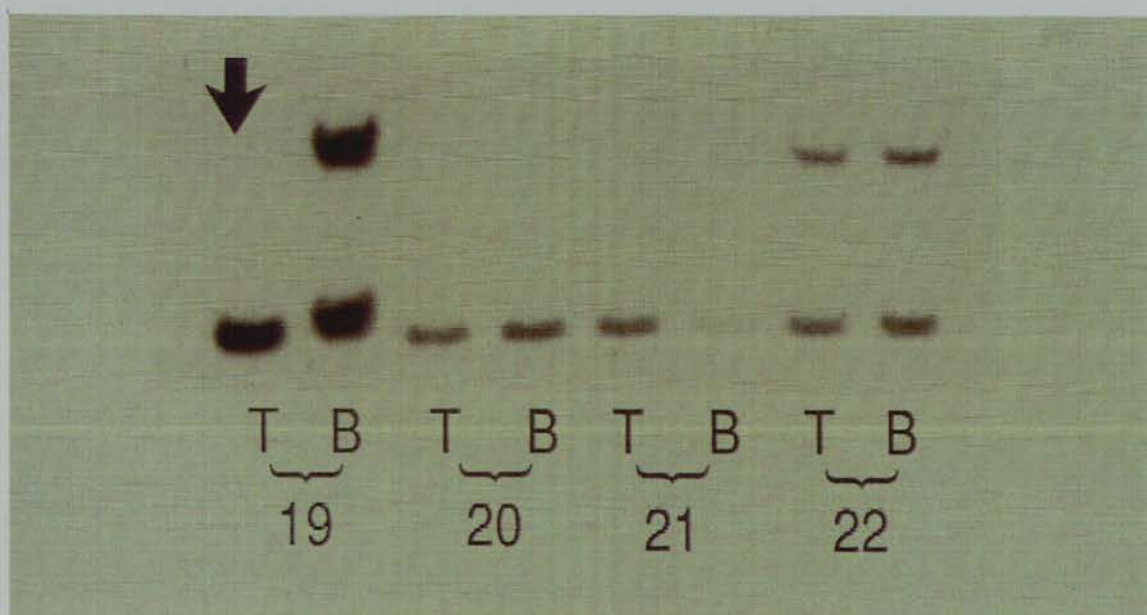


Fig. 3 Autoradiograph showing a typical set of results on RFLP analysis. Case 19 is heterozygous and therefore informative as two alleles are distinguishable in the constitutional DNA (19B). The corresponding tumour DNA (19T) shows loss of heterozygosity (LOH) (arrowed). Cases 20 and 21 are homozygous and hence uninformative as two distinct alleles cannot be identified in the constitutional DNAs (20B and 21B), while Case 22 is heterozygous and hence informative but shows no LOH.

TABLE IV Frequency of LOH with 22 RFLP markers in 20 Cervical Carcinomas

CHR. LOCUS		PROBE (ENZYME)	A/B	CASE NOS.
3p	3pter-p21	pEFD 145 (Rsa I)	1/4	6
5q	5q21	pL 5.62 (Bgl II)	1/2	14
5q	5q21-22	MC 5.61 (Msp I)	0/8	-
5q	5q21	pEF 5.44 (Msp I)	2/11	13 & 16
5q	5q21-22	YN 5.48 (Msp I)	3/13	4, 5 & 16
5q	5q21	MN 2.3 (Msp I)	0/2	-
5q	5q15-21	ECB 27 (Bgl II)	0/6	-
		Any 5q	5/16	
8q	8q22-23	TL 11 (Hind III)	1/7	13
11p	11p15.5	pEJ6.6 (BamH I)	1/15	13
11p	11pter-11p15.4	PTH (Pst I)	1/14	1
11p	11p15.4	Calcitonin (Taq I)	3/10	4, 5 & 16
11p	11p13	FSH β (Hind III)	0/4	-
		Any 11p	5/19	
13q	13q14	**p68RS 2.0 (Rsa I)	0/14	-
17p	17p13.3	**YNZ 22.1 (Taq I)	1/12	13
17p	17p13.3	**YNH 37.3 (Taq I)	1/14	13
17p	17p11.2-cen	pEW 301 (Taq I)	1/10	16
17p	17p12-13	pBHP 53 (BamH I)	1/9	14
17p	17p13	pMCT 35.1 (Msp I)	1/11	16
17p	17p13	C3068 (Hae III)	0/15	-
		Any 17p	3/20	
17q	17q23-25.3	**pTHH 59 (Taq I)	0/13	-
18q	18q21.3	pBV 15.65 (Msp I)	1/5	16
18q	18q21	SAM 1.1 (EcoR I)	3/12	7, 8 & 16
		Any 18q	3/12	

A = No. of cases showing LOH; B = No. of informative cases

** = Probe detecting VNTR (Variable No. of Tandem Repeats) sequence

CHAPTER 3

DNA FINGERPRINTING

Two minisatellite probes, 33.15 and 33.6 were used to detect the DNA fingerprints of 30 tumour blood pairs. A fingerprint was considered as interpretable if more than 10-12 corresponding distinct hypervariable fragments could be distinguished in the 4-20kb range in the fingerprints of both the tumour and the blood DNA. In some cases, the bands from either the blood or the tumour DNA appeared to become substantially less intense or distinct towards the 20kb end of the spectrum. In these cases, comparison with its corresponding fingerprint often proved difficult, as it became less easy to interpret band alterations reliably at this end of the range.

The resultant autoradiographs from these analyses revealed interpretable DNA fingerprints in 24 cases examined with minisatellite probe 33.15, and 24 cases examined with minisatellite probe 33.6. There were interpretable DNA fingerprints with both probes in 23 cases - 4 colonic carcinomas, 3 breast carcinomas, 4 ovarian epithelial carcinomas, 1 small bowel leiomyosarcoma, 1 mucinous cystadenocarcinoma of the appendix (examples of tumour types known to show high levels of allele loss) and 10 cervical carcinomas - and the results are shown in Table V.

In 7 cases, (1 colonic, 2 breast, 1 ovarian and 3 cervical carcinomas) the DNA fingerprint of the blood and/or tumour DNA detected with probe 33.15 and/or probe 33.6 was uninterpretable. This occurred either because of DNA overloading, which resulted in a high

background that completely obscured the hypervariable fragments; or more commonly because the majority of bands in the track were either very faint or completely absent, presumably the result of insufficient DNA or inefficient DNA digestion or probe hybridisation.

In the majority of cases (18/24) which gave interpretable results with probe 33.15, the tumour and constitutional DNA fingerprints were indistinguishable regardless of tumour type. The same held true for the DNA fingerprints produced by minisatellite probe 33.6 in that the tumour and constitutional DNA fingerprints were identical in 20 of 24 cases. Indistinguishable tumour/blood DNA fingerprints were obtained with both minisatellite probes in a total of 15 of 23 cases (Table V). Alterations in tumour DNA fingerprints comprised loss of band(s), acquisition of novel hypervariable fragments (bands), an increase or decrease in band intensity, or combinations thereof (Fig. 4).

Probe 33.15

In 3 of the cases examined with probe 33.15 (1 colonic and 2 ovarian carcinomas) there were shifts in the relative intensities of hypervariable fragments. The colonic carcinoma showed a relative decrease in intensity in a single band, while both ovarian carcinomas showed increases in band intensity in bands adjacent to one that had been deleted. Five cases showed evidence of loss of hypervariable fragments from the tumour DNA fingerprint. Two ovarian carcinomas showing losses have already been mentioned; a third ovarian carcinoma showed evidence of loss of 2 fragments; while 2 cervical carcinomas (Case Nos. 7 and 13) also displayed evidence of band deletion. Cervical tumour Case No. 7 lost 6 hypervariable fragments in association with the acquisition of 3 novel fragments; while cervical tumour Case No. 13 lost 1 fragment.

Probe 33.6

Shifts in hybridisation band intensity were also detected with minisatellite probe 33.6, which detects hypervariable fragments derived from a different set of loci from those detected by probe 33.15. A colonic carcinoma showed reduced intensity of one band at the 20kb end of the range. However, this observation is of uncertain reliability as, in this case, the tumour-derived fragments all exhibited diminished intensities toward the 20kb end of the fingerprint. Two breast carcinomas displayed increased intensity, each of a single hypervariable fragment; and one cervical carcinoma (Case No. 7) showed diminished intensity of one band in association with the loss of 4 fragments, and the acquisition of one novel fragment.

In summary (Table V), probe 33.15 detected alterations in the tumour DNA fingerprints of 1 of 4 colonic carcinomas, 3 of 5 ovarian carcinomas, 2 of 10 cervical carcinomas, and none of 3 breast and 2 small bowel cancers, when compared to that of their respective constitutional DNA fingerprints. For probe 33.6, 1 of 5 colonic, 2 of 3 breast, 1 of 10 cervical carcinomas and none of 4 ovarian and 2 small bowel cancers showed alterations in their tumour DNA fingerprints. A total of 9 of the 25 cases which generated interpretable results with probes 33.15 and/or 33.6 demonstrated alterations in their tumour DNA fingerprint. Of these, 2 out of 10 were cervical carcinomas, while 7 out of 15 were cancers of non-cervical origin.

It is perhaps interesting to note that cervical carcinomas Case Nos. 13 and 16, both of which showed multiple allele losses on RFLP analysis, did not show tumour DNA fingerprint alterations. While case no. 13 (LOH detected at 5 chromosomal loci by RFLP

analysis) showed one loss in its tumour DNA fingerprint with probe 33.15 and no alterations with probe 33.6; the tumour and blood DNA fingerprints of Case No. 16 (LOH detected at 7 chromosomal loci by RFLP analysis) were identical with probe 33.15 and probe 33.6.

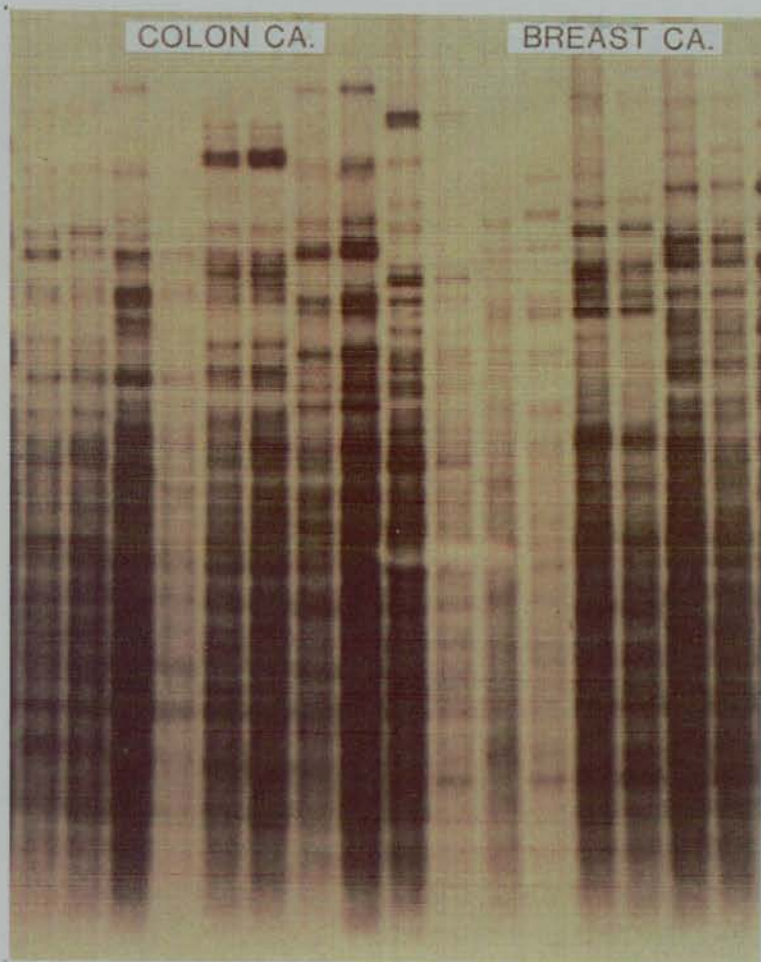


Fig 4a DNA fingerprints of colonic and breast carcinoma tumour/blood pairs with minisatellite probe 33.15 showing indistinguishable tumour and constitutional DNA fingerprints in most tracks. Tumour and blood DNA are in adjacent tracks, L → R track 1 corresponds to the tumour DNA fingerprint of colon carcinoma No. 2 with its blood DNA fingerprint to the right in track 2.

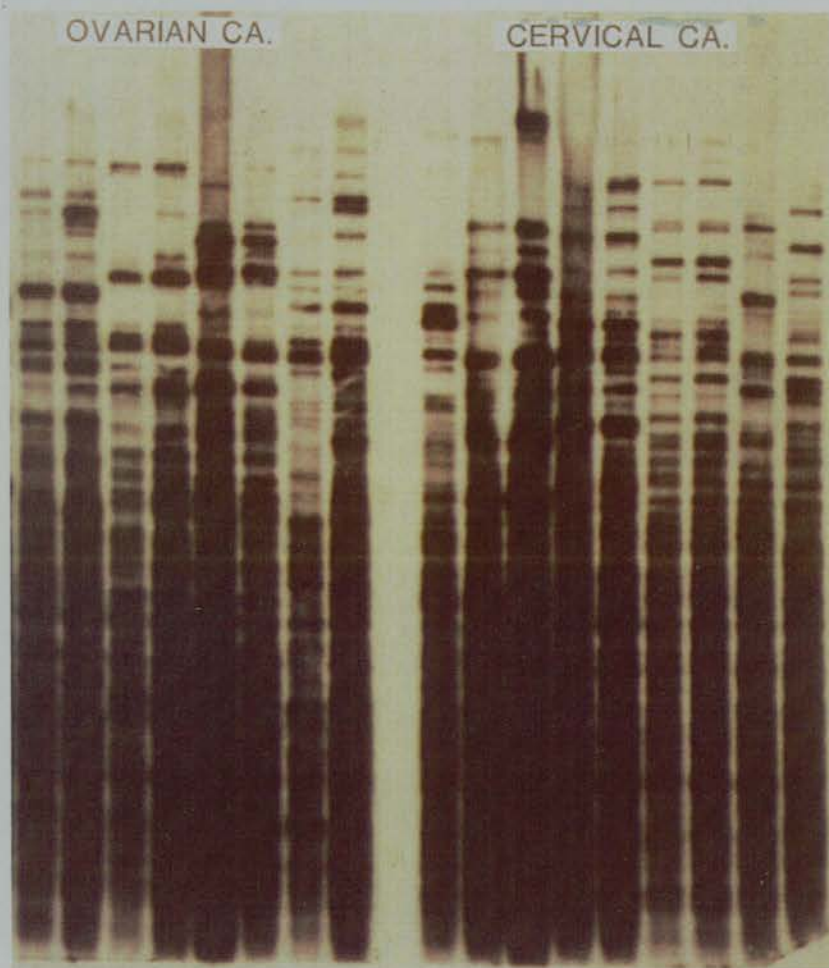


Fig 4b DNA fingerprints of ovarian and cervical carcinoma tumour/blood pairs with minisatellite probe 33.6 showing indistinguishable tumour and constitutional DNA fingerprints in most tracks. Tumour and blood DNA are in adjacent tracks, L → R track 1 corresponds to the tumour DNA fingerprint of ovarian carcinoma No. 2 with its blood DNA fingerprint to the right in track 2

TABLE V

**Tumour vs. Constitutional DNA - Alterations in Tumour DNA
Fingerprints produced with Minisatellite Probes 33.15 and 33.6**

NO.	TUMOUR TYPE (*)	DNA FINGERPRINT ALTERATIONS DETECTED	
		PROBE 33.15	PROBE 33.6
1	Colon	N/I	↓
2	Colon	-	-
3	Colon	-	-
4	Colon	↓	-
5	Colon	-	-
6	Breast	-	↑
7	Breast	-	-
8	Breast	-	↑
9	Ovary	-	N/I
10	Ovary	d, 2↑	-
11	Ovary	-	-
12	Ovary	d, 2↑	-
13	Ovary	2d	-
14	Small bowel	-	-
15	Appendix	-	-
16	Cervix (2)	-	-
17	Cervix (3)	-	-
18	Cervix (6)	-	-
19	Cervix (7)	6d, 3n	4d, n, ↑
20	Cervix (9)	-	-
21	Cervix (12)	-	-
22	Cervix (13)	d	-
23	Cervix (16)	-	-
24	Cervix (17)	-	-
25	Cervix (19)	-	-

* = Case no. (1-55) of cervical carcinomas collected

N/I = Not interpretable

↓ = Diminished intensity of hypervariable fragment(s)

↑ = Increased intensity of hypervariable fragment(s)

d = Deletion or loss of hypervariable fragment(s)

n = Acquisition of novel hypervariable fragment(s)

CHAPTER 4

HPV STATUS OF TUMOURS

1. HPV STATUS OF TUMOURS EXAMINED FOR ALLELE LOSS

For the initial 20 cervical carcinomas examined for allele loss, the HPV status with respect to types 6, 11, 16, 18, and 33 was determined by PCR analysis, and the state of the virus within the genome (integrated vs episomal) was determined by Southern blotting. Table VI lists the chromosomal arms showing allele losses in each tumour, and indicates whether the viral DNA was found to be integrated or episomal.

As expected, HPV DNA was detectable (Fig. 5) in 75% (15/20) of these tumours, and was found to be integrated rather than episomal in the majority (9/12) of the HPV-positive tumours from which interpretable results were obtained. HPV types 16 and 18 were detected most frequently (type 16 only in 9 tumours; type 18 only in 3 tumours; and both types 16 and 18 in 2 tumours), with a total of 14 of the 15 HPV-positive tumours being positive for HPV 16 and/or 18. HPV 33 was detected in only one tumour, while neither HPV 6 nor HPV 11 was detected in any of them. Five (25%) of the carcinomas were negative for all of the HPV types tested.

Neither viral presence nor its integration correlated with the LOH observed at any specific chromosomal region, nor with the frequency of allele losses seen in any tumour.

2. HPV ANALYSIS OF ALL CERVICAL TUMOURS EXAMINED

Fifty-five cervical carcinomas were analysed for the presence of HPV types 16, 18, and 33. Analyses for HPV types 6 and 11 were also performed on tumours 1-20, and the results are shown in Table VI. Given the association of HPV types 6 and 11 primarily with benign rather than malignant cervical lesions, and the support provided by the negative results of analyses for these types in the first 20 tumours, analysis of the remaining 35 carcinomas (cases 21-55) was restricted to HPV types 16, 18, and 33.

The HPV status of each of the 55 carcinomas examined is shown in Table VII. HPV types 16, 18, or 33 were detected in 71% (39/55) of the carcinomas analysed. Sixteen tumours were negative for all three HPV types and, as in the other 39 tumours, the efficiency of the PCR reactions was demonstrated for these cases by successful PCR amplification of their ras sequences. Five of the sixteen negative cases had been amongst those analysed for HPV 6 and 11, for which they had also been negative. There were 18 tumours in which only HPV 16 was detected, 10 in which only type 18 was detected, and 10 cases in which both types 16 and 18 were identified. Over the entire series of 55 cases, only one carcinoma showed HPV 33 positivity.

Of the 6 "glandular" (3 adeno-, 3 adenosquamous) carcinomas represented, all but one were positive for HPV 18. The exception was negative for all the other HPV types examined. By comparison, HPV 18 on its own accounted for HPV positivity in only 7 of 33 HPV positive squamous cervical carcinomas. Both HPV 16 and 18 were detected in 2 of the glandular cancers, while 8 out of 10 (80%) of the tumours positive for both of these virus types were squamous in type.

The presence of HPV 16, 18, or 33 DNA appeared to bear no relationship to patient age, the tumour stage at presentation, or its degree of differentiation.

TABLE VI**Allele Losses and HPV Types and State in Cervical Carcinomas Studied**

CASE NO	HISTOLOGICAL TYPE	FIGO STAGE	LOCATION OF ALLELE LOSS	HPV TYPE PRESENT(*)
1	A	IIb	11p	-
2	S	Ib	-	16(i)
3	AS	Ib	-	18(e)
4	S	Ib	5q, 11p	16(i) & 18(i)
5	S	IIIb	5q, 11p	16(e)
6	S	Ib	3p	-
7	S	Ib	18q	16
8	S	Ib	18q	33
9	S	IIIa	-	-
10	A	Ib	-	18(e)
11	S	Iva	-	16(i)
12	S	IIb	-	16(i)
13	S	IIb	5q, 8q, 11p, 17p	16(i) & 18(e)
14	S	Ib	5q, 17p	-
15	S	Ib	-	-
16	S	Ib	5q, 11p, 17p, 18	16(i)
17	S	Ib	-	16(i)
18	S	IIb	-	18(i)
19	S	IIb	-	16(i)
20	S	IIIb	-	16

S = Squamous; AS = Adenosquamous; A = Adenocarcinoma

(*): (i) = integrated HPV DNA; (e) = episomal HPV DNA

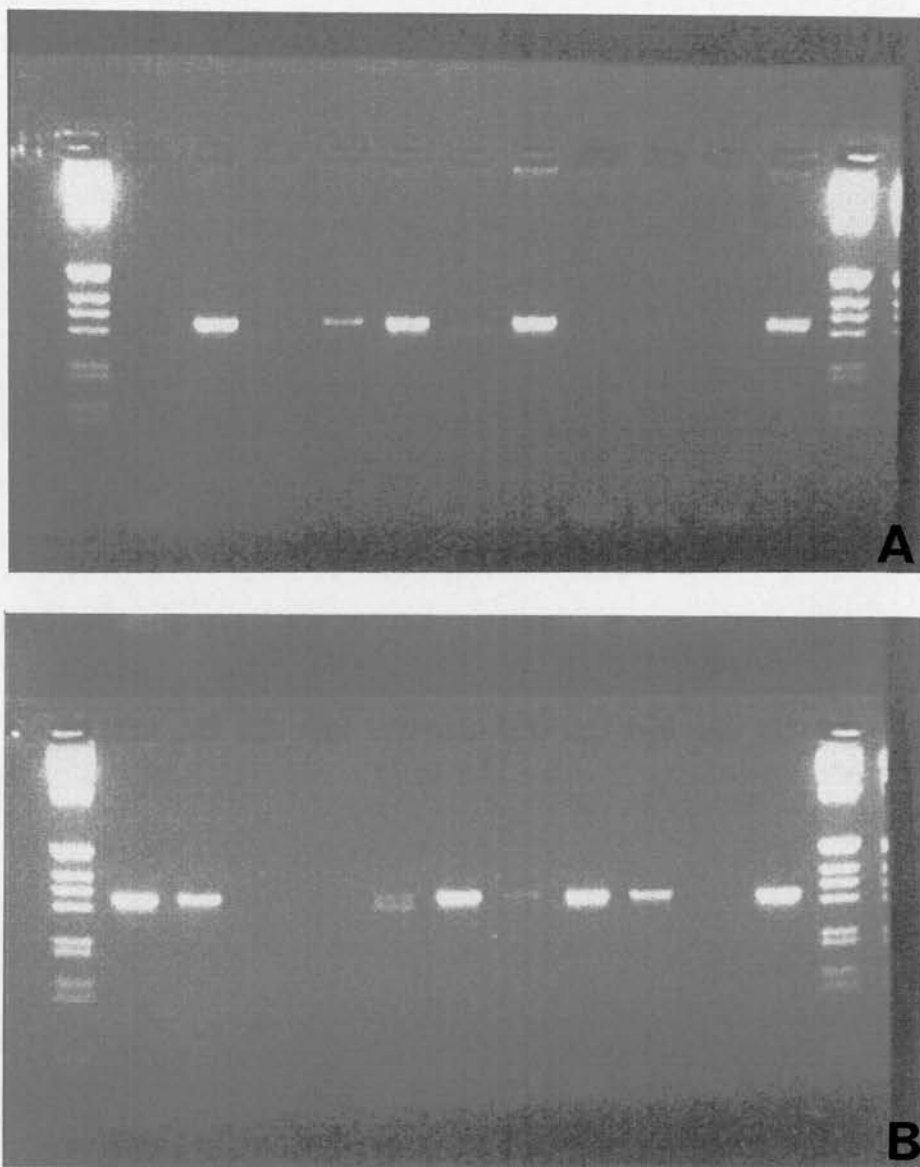


Fig. 5. UV transilluminated gels following gel electrophoresis of products from PCR amplification of tumour DNA from Cases 1 - 11 (A), and Cases 12 - 20 (B) with HPV 16 PCR primers. Fig. 5A L → R = Cases 1 - 11. Fig. 5B L → R = Cases 12 - 20, followed by the negative control (distilled water) with the positive control (HPV 16 plasmid DNA) in the rightmost track. Bands confirm the presence of HPV 16. 1kb lambda ladders used for band size comparison can be seen in the outermost tracks on either side of each gel.

TABLE VII
Clinicopathological features and HPV status of 55 cervical carcinomas studied

CASE NO	AGE	HISTOLOGICAL TYPE	GRADE	FIGO STAGE	HPV TYPE
1	58	ADENOCARCINOMA	P	IIB	-
2	31	SQUAMOUS CARCINOMA	M/P	IB	16
3	23	ADENOSQUAMOUS CARCINOMA	NR	IB	18
4	43	ADENOCARCINOMA	W	IB	16 & 18
5	67	SQUAMOUS CARCINOMA	M	IIIB	16
6	41	SQUAMOUS CARCINOMA	NR	IB	-
7	37	SQUAMOUS CARCINOMA	P	IB	16
8	30	SQUAMOUS CARCINOMA	M	IB	33
9	34	SQUAMOUS CARCINOMA	P	IIB	-
10	36	ADENOCARCINOMA	NR	IB	18
11	32	SQUAMOUS CARCINOMA	M	IVA	16
12	69	SQUAMOUS CARCINOMA	P	IIB	16
13	62	SQUAMOUS CARCINOMA	M	IIB	16 & 18
14	25	SQUAMOUS CARCINOMA	P	IB	-
15	61	SQUAMOUS CARCINOMA	NR	IB	-
16	41	SQUAMOUS CARCINOMA	M	IB	16
17	41	SQUAMOUS CARCINOMA	NR	IB	16
18	70	SQUAMOUS CARCINOMA	P	IIB	18
19	54	SQUAMOUS CARCINOMA	W	IIB	16
20	67	SQUAMOUS CARCINOMA	NR	IIIB	16
21	50	ADENOSQUAMOUS CARCINOMA	P	IIB	16 & 18
22	36	SQUAMOUS CARCINOMA	NR	IB	16
23	39	SQUAMOUS CARCINOMA	NR	IIA	16
24	63	SQUAMOUS CARCINOMA	NR	IB	16
25	69	SQUAMOUS CARCINOMA	NR	IIA	-
26	57	SQUAMOUS CARCINOMA	M	IB	16
27	47	SQUAMOUS CARCINOMA	NR	IIIB	16 & 18
28	43	SQUAMOUS CARCINOMA	NR	IIB	16 & 18
29	69	SQUAMOUS CARCINOMA	NR	IIB	-
30	61	SQUAMOUS CARCINOMA	NR	IIB	-

TABLE VII continued

CASE NO	AGE	HISTOLOGICAL TYPE	GRADE	FIGO STAGE	HPV TYPE
31	40	SQUAMOUS CARCINOMA	NR	IB	16
32	31	SQUAMOUS CARCINOMA	NR	IB	18
33	25	SQUAMOUS CARCINOMA	NR	IB	18
34	62	SQUAMOUS CARCINOMA	NR	IB	18
35	61	SQUAMOUS CARCINOMA	NR	IIB	-
36	57	SQUAMOUS CARCINOMA	P	IIIA	-
37	60	SQUAMOUS CARCINOMA	M	IIA	16 & 18
38	53	SQUAMOUS CARCINOMA	P	IIIB	16 & 18
39	63	SQUAMOUS CARCINOMA	M	IIIB	18
40	43	SQUAMOUS CARCINOMA	NR	IB	18
41	34	SQUAMOUS CARCINOMA	M	IB	16 & 18
42	72	SQUAMOUS CARCINOMA	NR	IIB	16 & 18
43	52	SQUAMOUS CARCINOMA	M/W	IIA	16 & 18
44	30	SQUAMOUS CARCINOMA	P	IB	-
45	32	SQUAMOUS CARCINOMA	P	IB	18
46	69	SQUAMOUS CARCINOMA	M	IIB	16
47	46	SQUAMOUS CARCINOMA	M/P	IIB	-
48	47	SQUAMOUS CARCINOMA	NR	IIA	-
49	40	SQUAMOUS CARCINOMA	P	IB	-
50	43	SQUAMOUS CARCINOMA	P	IIB	16
51	51	ADENOSQUAMOUS CARCINOMA	P	IIB	18
52	66	SQUAMOUS CARCINOMA	M/P	IIB	16
53	54	SQUAMOUS CARCINOMA	NR	IVA	-
54	42	SQUAMOUS CARCINOMA	M	IB	-
55	67	SQUAMOUS CARCINOMA	NR	IIA	16

P = poorly differentiated; M = moderately differentiated; W = well differentiated; NR = not recorded

CHAPTER 5

p53 ANALYSES

1. SCREENING FOR p53 MUTATIONS BY PCR FOLLOWED BY DGGE; AND SEQUENCING OF MUTATIONS

For each tumour, five regions of the p53 gene (the four hot-spot regions (HSRs) A-D and a fifth region, for convenience denoted as the "E" region (Chapter 5 Section 2)) were amplified by polymerase chain reaction (PCR). Of the 55 cervical carcinomas collected, there was sufficient DNA to permit successful amplification of all 5 regions in 47 cases. Amongst the remaining 8 tumours, there were 4 in which amplified products were obtained from only 1-4 of the regions; while in the other 4 cases there was insufficient DNA, and successful amplification was not achieved in any of the 5 regions (A-E) analysed.

Irrespective of which of the five primer pairs was being used, the negative control included in each PCR run consistently showed no detectable product band on UV transillumination of the ethidium bromide stained gel, after electrophoresis. This indicated that no detectable primer dimer formation nor sample DNA contamination had occurred.

The products of all successful amplifications (band of appropriate size detectable on UV transillumination of the ethidium bromide stained gel, following electrophoresis) (Fig. 6) were screened for p53 mutations by denaturing gradient gel electrophoresis (DGGE).

For each of the five positive controls, amplification of the region in which a p53 mutation had previously been identified, yielded a product which on DGGE migrated at a different rate from its unmutated counterpart.

Denaturing gradient gel electrophoresis (DGGE) of each of the products from PCR amplification of regions A-D of the p53 gene, using 4 primer pairs, revealed a single band in each of 46 of the 47 cervical carcinomas analysed. For each region, each of these 46 tumour samples yielded a PCR product with the same DGGE mobility as that of the normal constitutional DNA, indicating the absence of mutation in regions A, B, C and D of the p53 gene in all but one of the 47 tumours examined.

Denaturing gradient gel electrophoresis of the PCR-amplified product of HSR B (exon 5's codons 155 to 185) in one cervical carcinoma (Case No. 53 - a stage IVa, HPV-negative, squamous carcinoma from a 54 year old patient) revealed a mutant band. This migrated at a slower rate than its normal counterpart, and could not be detected in the constitutional (blood) DNA (Fig. 7). Subsequent sequencing of the tumour DNA from this region confirmed the presence of a CGC → TGC transition at codon 175. This transition results in a change in the encoded amino acid from arginine to cysteine.

In 4 further tumours, there were amplified products from only selected regions (1 from regions C, D and E; 1 from regions A and C; 1 from regions A, B, C and E; and 1 from region A only), and DGGE of these products also revealed a single band from each tumour.

Of the 49 carcinomas amplified with the "E" primers, two (Cases nos. 1 and 52) produced

amplified fragments that migrated at a different rate from that of their normal counterparts (Fig. 8). DGGE of the "E"-primer amplified fragments of DNA from these tumours, and the constitutional (blood) DNA from the corresponding individuals, both revealed heterozygosity, in that two distinct bands could be distinguished in each sample (Fig. 9).

Sequencing of this region ("E") of the p53 gene of the tumour and the constitutional DNA, in both cases, demonstrated the presence of a CGA → CGG transition at codon 213, confirming the findings on DGGE. It was in fact shown to be a silent mutation, as both sequences encode the same amino acid, arginine. One of these two tumours in which this silent mutation was detected was negative for the 5 HPV types analysed, namely types 6, 11, 16, 18, and 33; while the other was positive for HPV 16.

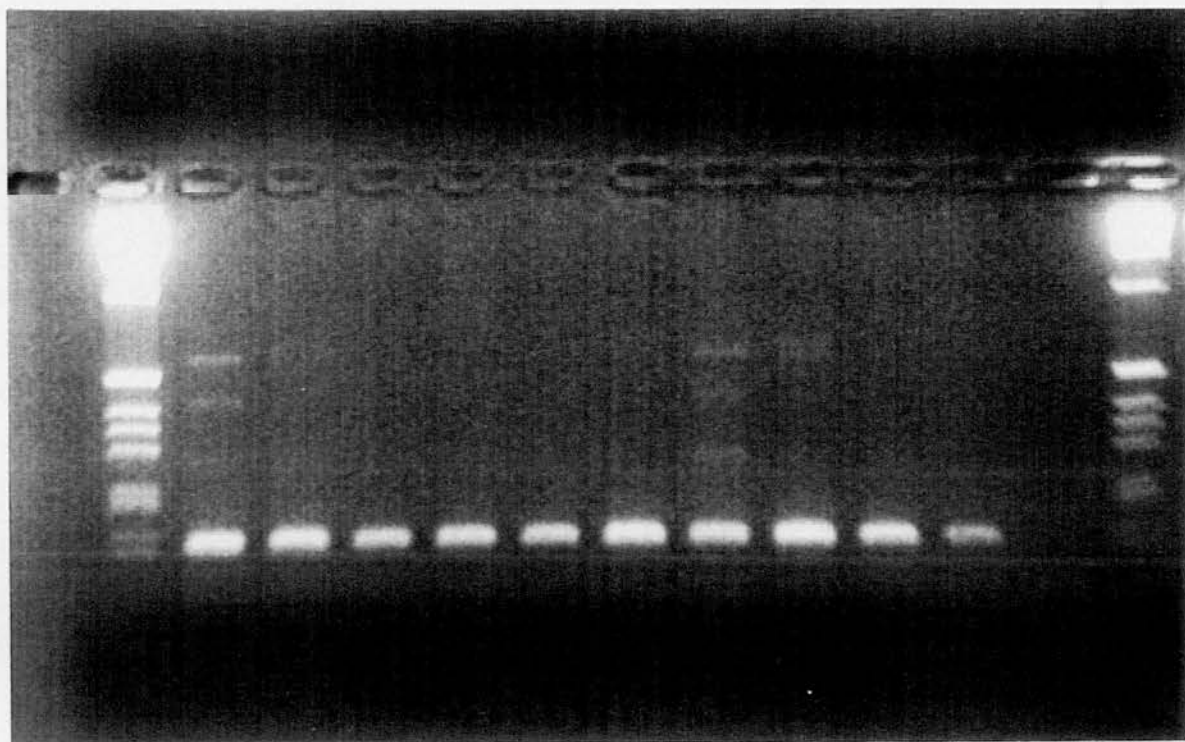


Fig. 6 UV transilluminated 3% agarose gel stained with ethidium bromide following electrophoresis of products from PCR amplification of DNA of cervical tumours with the "A" primers, which amplified HSR A of the p53 gene. Detectable PCR fragments in each lane indicate successful amplification of this region of the p53 gene in each tumour. The 1kb lambda markers at the extreme left and right permit band size comparison. There is no detectable band in the lane immediately to the left of the right 1kb ladder as this is the negative control in which distilled water was substituted for template DNA, and confirms the absence of any primer/dimer formation or contamination of reagents.

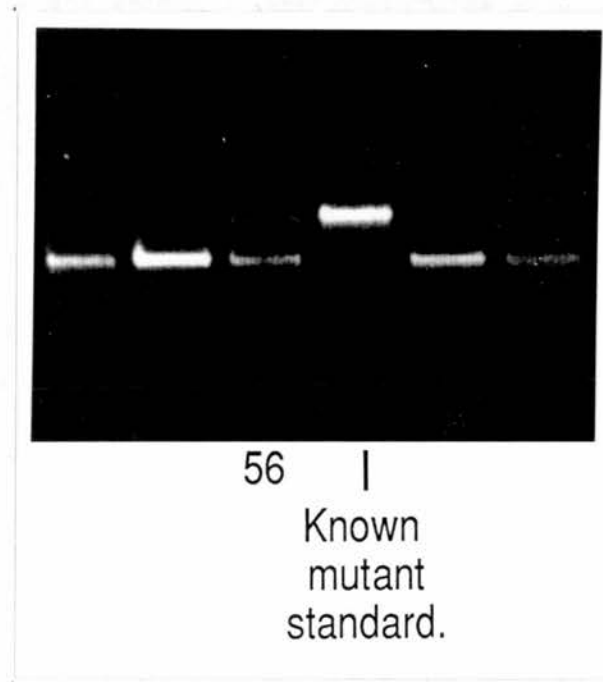


Fig.7 DGGE of PCR amplified products of HSR B (exon 5's codons 155-185) of the p53 gene in cervical tumours and positive control. Case No. 53 (shown as 56 in photograph) shows a mutant band similar to that of the known mutant standard in the adjacent track. Single bands from tumours without such mutations are seen in the other 4 unlabelled tracks.

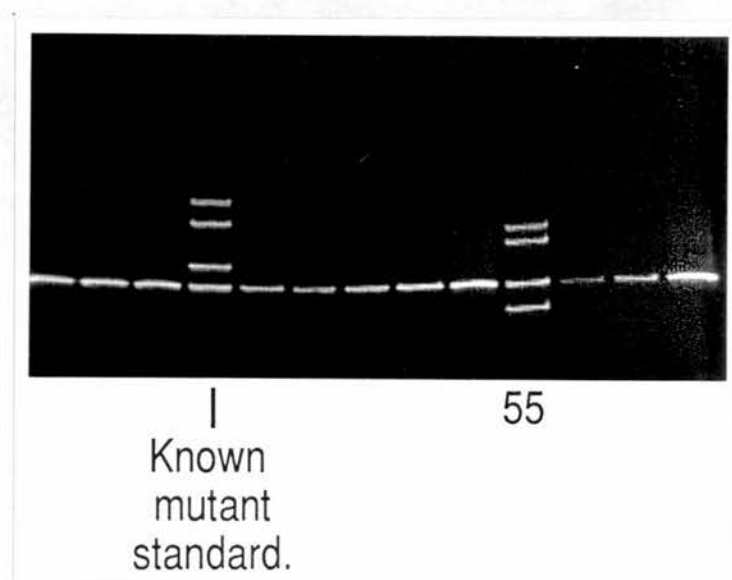


Fig. 8 DGGE of the "E" primer amplified fragments (exon 6's codons 193-218) of the p53 gene in cervical tumours and positive control. Case No. 52 (shown as 55 in photograph) shows mutant bands similar to those of the known mutant standard. Single bands from tumours without such mutations are seen in the unlabelled tracks.

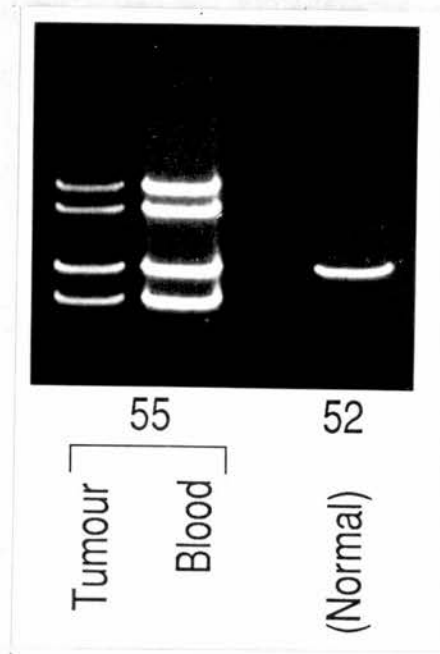


Fig. 9 DGGE of the "E" primer amplified fragments (exon 6's codons 193-218) of the p53 gene in cervical tumour and its corresponding blood (constitutional) DNA from Case No. 52 (shown as 55 in photograph), and DNA from cervical tumour Case No. 49 (shown as 52 in photograph). The presence of 4 distinct bands in both the tumour and blood DNA of Case No. 52 (55 in photograph) suggests the presence of a constitutional polymorphism rather than a mutation. A normal band (neither polymorphism nor mutation present) is shown in Case No. 49 (52 in photograph).

2. p53 IMMUNOHISTOCHEMICAL ANALYSIS

2.1. CASES ANALYSED

Tumour tissue from 46 of the 55 freshly collected carcinomas was available for p53 immunocytochemical analysis. In addition, biopsy specimens from a further 11 carcinomas were obtained from archival material for this analysis. Other specimens, obtained from archival material comprised cervical biopsies from 43 patients with CIN or a diagnosis of cervical glandular atypia. In addition, cervical tissue was obtained fresh from a further 14 patients undergoing hysterectomy for benign non-cervical uterine conditions. In total, cervical biopsies obtained from 115 patients were analysed immunohistochemically for p53 expression. The distribution of the histological appearances recognised is shown below.

TABLE VIII

p53 Immunohistochemistry: histological Appearances in 115 Cervical Biopsies

HISTOLOGY	NO. OF CASES
Squamous carcinoma	51
Adenocarcinoma	3
Adenosquamous carcinoma	3
CIN3	46
CIN 1 or 2	24
Koilocytosis	22
Glandular atypia / adenocarcinoma-in-situ	3
Benign metaplastic changes in glands	9
Normal squamous epithelium	53
Normal endocervical glands	62

Of the 115 specimens, 36 had been fixed in formalin, so that for these, results are available only with the p53 antibody MAb DO-7. The other 79 specimens had been fixed in PLPD, and hence results of staining with both p53 antibodies (MAb DO-7 and PAb 1801) have been generated.

2.2. p53 IMMUNOHISTOCHEMICAL STAINING OF CONTROL TISSUES

Both antibodies, PAb 1801 and DO-7, produced specific nuclear staining in the positive control colonic carcinoma tissue fixed in PLPD (Figs. 10 and 11). In sections taken from this specimen, areas of carcinoma showed clear nuclear localisation of p53 product, while normal colonic mucosa was negative.

For tissue sections fixed in PLPD, antibody DO-7 produced superior staining to PAb 1801, being crisper and more intense, although the distribution of positively stained cells was similar.

PAb 1801 was not used on formalin-fixed tissues. The antibody DO-7 produced patchy nuclear staining in formalin-fixed tissues, which, although unequivocally positive, was inferior to that obtained with PLPD-fixed tissues.

2.3. p53 IMMUNOHISTOCHEMICAL STAINING OF CERVICAL TISSUES

Positive staining was found in 13 out of 115 cervical biopsy specimens, with only 8 of the 57 carcinomas examined showing any positive staining. In each case staining was focal in distribution, with only occasional nuclei showing positive staining, in a background population of entirely negative cells (Fig 12). Details of positively staining cases are shown in Table IX.

Nine of the positive cases occurred in PLPD-fixed specimens, and 3 of these showed positive staining only with PAb 1801. Two of the latter were from patients who had undergone hysterectomy for benign uterine disease, and with PAb 1801, showed staining in endocervical glands, in one of which there was tubo-endometrial metaplasia. The other PAb 1801-positive specimen was derived from a moderately differentiated, stage IIb squamous carcinoma from a 69 year old patient (Case No. 46).

One of the positive PLPD-fixed cases (Case No. 52) was a stage IIb poorly to moderately differentiated squamous carcinoma, from a 66 year old patient, which displayed relatively extensive staining with both PAb 1801 and MAb DO-7 (Fig. 13). The other 5 positive PLPD-fixed cases were all invasive squamous carcinomas (Case Nos. 44, 45, 49, 50 and 53) and exhibited positive staining only with MAb DO-7. Three of them were poorly differentiated stage Ib carcinomas from patients in the 30-40 years age group; one was a poorly differentiated stage IIb carcinoma from a 43 year old patient; while the other was a stage IVa carcinoma from a 54 year old patient.

Four of the formalin-fixed specimens showed focal positive staining. Two of these cases demonstrated CIN2, one CIN3, and one a poorly to moderately differentiated, stage IIIb invasive squamous carcinoma from a 67 year old patient (Case No. 5).

In comparison with the strongly positive staining seen in the controls, staining of cervical tissues was always faint and more sparsely distributed.

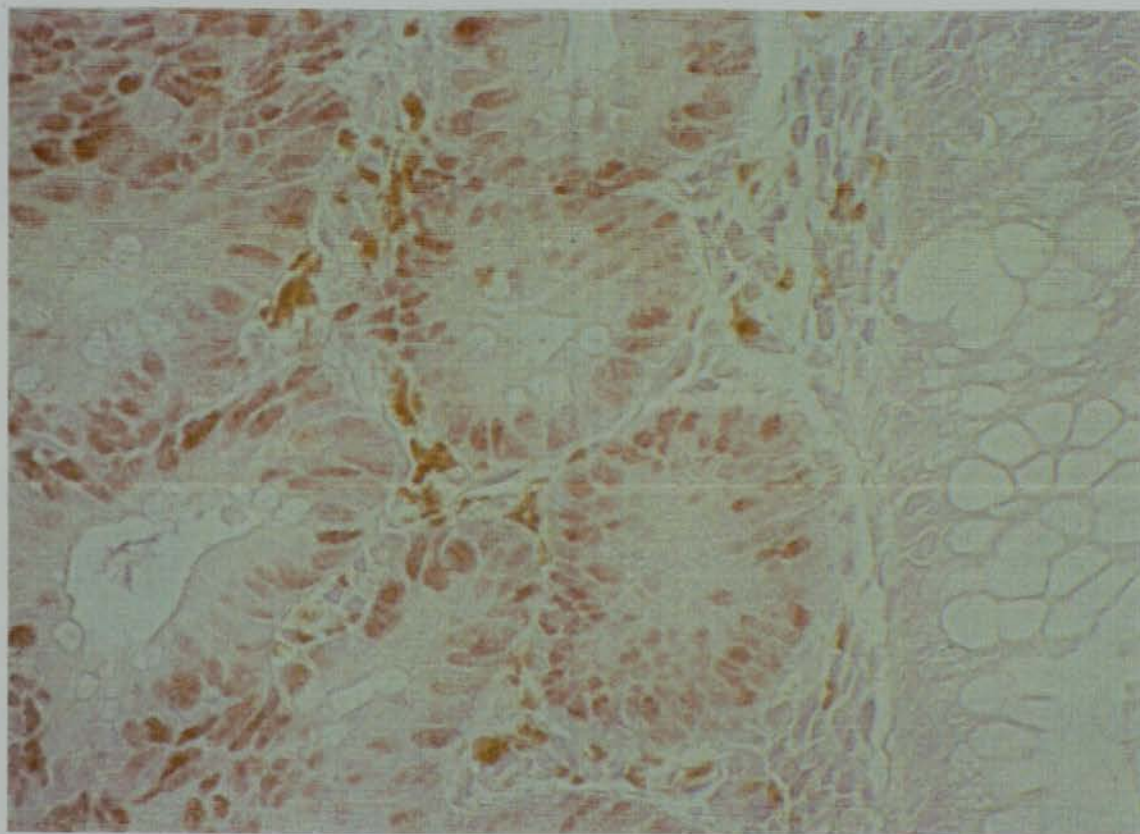


Fig. 10 Immunohistochemical staining with p53 antibody PAb1801 in a section from the PLPD-fixed positive control colonic carcinoma known to possess mutated p53. Clear nuclear localisation of stain is seen in cells of malignant colonic epithelium, but adjacent normal colonic epithelium is unequivocally negative.

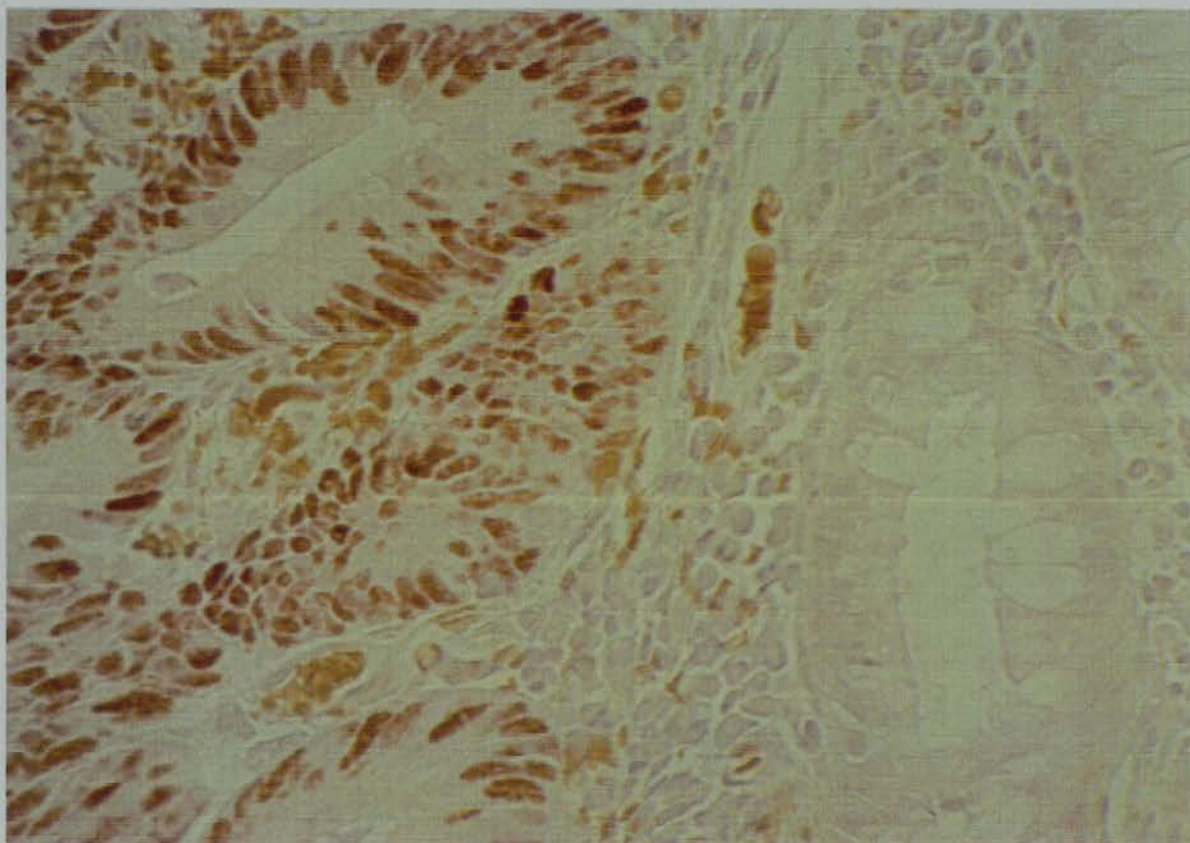


Fig. 11 Immunohistochemical staining with p53 antibody MAb DO7 in a section from the same PLPD-fixed positive control colonic carcinoma known to possess mutated p53 as shown in Fig. 10. Staining is superior to that obtained with PAb1801 (Fig. 10), being crisper and more intense, but shows a similar distribution of positively-stained cells, with clear nuclear localisation of stain seen in cells of malignant colonic epithelium with adjacent normal colonic epithelium unequivocally negative.

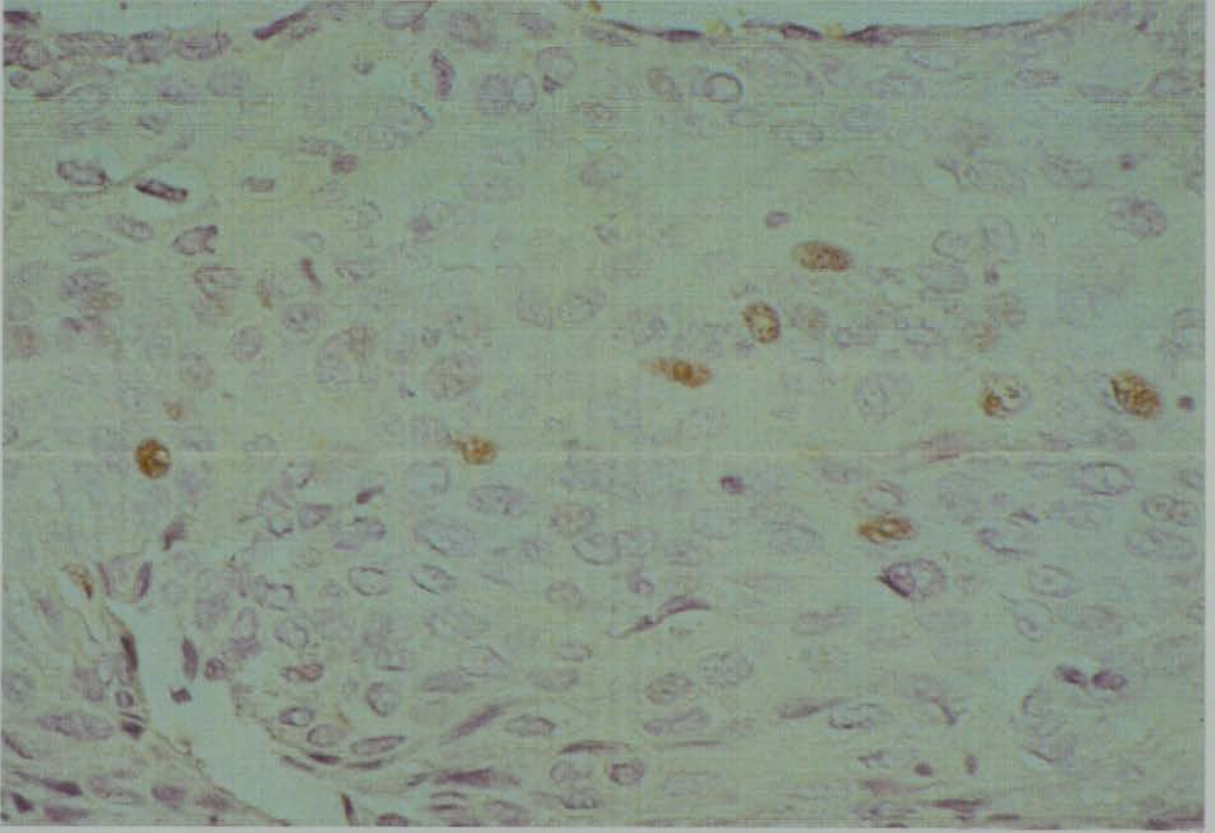


Fig. 12 Immunohistochemical staining with p53 antibody MAb DO7 in a section of formalin-fixed cervical epithelium showing CIN 3. In comparison to the staining in positive controls, positively stained nuclei are sparsely distributed, less intensely stained and detected in a background of entirely negative cells.

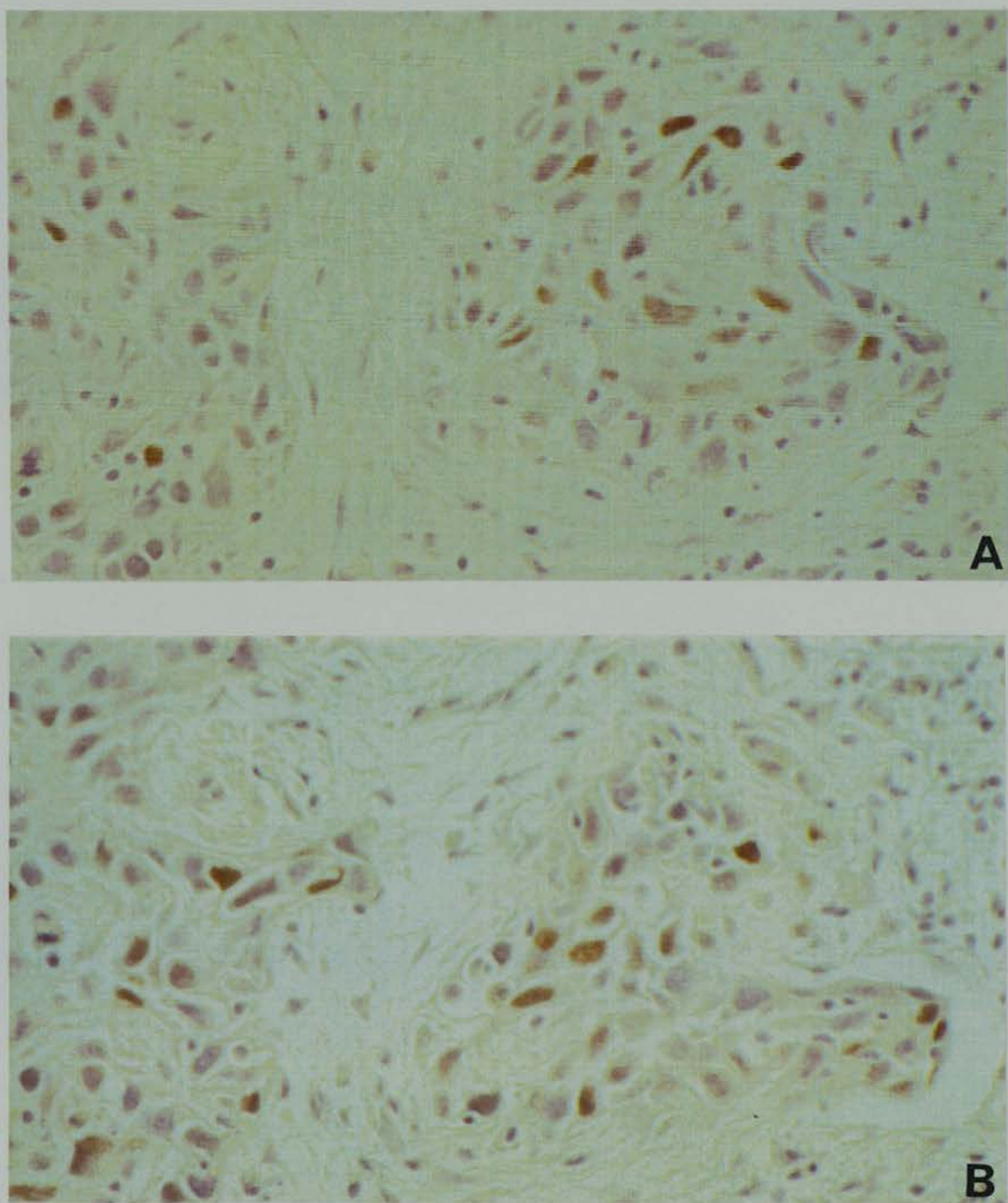


Fig. 13 Immunohistochemical staining with p53 antibody PAb1801 (Fig. 13A) and MAb DO7 (Fig. 13B) in serial sections from PLPD-fixed cervical carcinoma Case No. 52 (an HPV 16 positive squamous carcinoma). Positive nuclear staining is seen with both antibodies.

TABLE IX

Clinicopathological features of specimens showing immunohistochemical p53 positivity

Squamous carcinomas (n=8)

Stage	Grade	Fixative	HPV Status	PAb 1801	MAb DO7
Iib	Moderate	PLPD	16	+	-
Iib	Poor-Mod	PLPD	16	+	+
Ib	Poor	PLPD	Negative	-	+
Ib	Poor	PLPD	18	-	+
Ib	Poor	PLPD	Negative	-	+
Iib	Poor	PLPD	16	-	+
IVa	Poor	PLPD	Negative	-	+
IIIb	Moderate	Formalin	16	Not tested	+

Non-carcinoma specimens (n=5)

Histology	Fixative	PAb 1801	MAb DO7
Normal endocervical glands	PLPD	+	-
Metaplastic endocervical glands	PLPD	+	-
CIN2	Formalin	Not tested	+
CIN2	Formalin	Not tested	+
CIN3	Formalin	Not tested	+

3. p53 MUTATIONS AND HPV STATUS

Of 11 HPV-negative tumours, in which all five p53 regions had been successfully amplified, only two (Cases Nos. 1 and 53) revealed p53 mutations on DGGE screening. Only one of these was indeed a somatic mutation (Case No. 53); the other (Case No. 1) was silent. Of the 36 HPV-positive tumours similarly analysed, only one revealed a silent mutation (Case No. 52), and no somatic mutations were detected.

Of the 8 cervical carcinomas exhibiting positive staining with PAb 1801 and/or MAb DO-7, 4 were positive for HPV 16; 1 was positive for HPV 18; and 3 were HPV-negative.

Of the 13 specimens which showed positivity with either one or both antibodies, the hot-spot regions of the p53 gene in samples of DNA from 8 of the invasive carcinomas had been amplified by PCR and screened for mutations by DGGE. Among these 8 carcinomas displaying immunohistochemical p53 positivity, only 2 showed mutations when screened by DGGE (Cases Nos. 52 and 53), and only one (Case No. 53) was a somatic mutation. Conversely, of the 3 tumours in which mutations were detected by PCR/DGGE (Cases Nos. 1, 52 and 53), only 2 showed immunohistochemical positivity with one (Case No. 53) (with MAb DO-7) or both (Case No. 52) (MAb DO-7 and PAb 1801) antibodies.

Of interest is the fact that tumour no. 52 (a stage IIb, poorly to moderately differentiated squamous carcinoma, from a 66 year old patient) which showed positivity with both antibodies, and in which a mutation (albeit silent) was detected in the "E" region of the p53 gene, was positive for HPV 16.

CHAPTER 6

ras p21 IMMUNOHISTOCHEMICAL ANALYSIS

1. CASE DISTRIBUTION

As antibody Y13 259 could be used for ras p21 immunohistochemical analysis only on PLPD-fixed tissue, the histological distribution of specimens stained with this antibody was somewhat different from that of specimens stained with antibodies to p53. Cervical biopsies from a total of 89 patients were examined for immunohistochemical expression of ras p21 protein, and all tissues had been fixed in PLPD. A spectrum of histological appearances was represented in the specimens analysed, and their distribution is tabulated below (Table X).

TABLE X

Histological Appearances in 89 Cervical Biopsies Examined Immunohistochemically for ras p21 expression

HISTOLOGY	NO. OF CASES
Squamous carcinoma	37
Adenocarcinoma	2
Adenosquamous carcinoma	2
CIN 3	32
CIN 1 and/or CIN 2	13
Koilocytosis	6
Glandular atypia / adenocarcinoma in-situ	3
Benign metaplastic changes in glands	10
Normal squamous epithelium	23
Normal endocervical glands	49

Seventy-eight cases coincided with those examined for immunohistochemical expression of p53 and included samples from 32 of the 55 freshly collected cervical carcinoma specimens and the 14 specimens collected from patients undergoing hysterectomy for benign non-cervical disease. The remaining 43 samples were colposcopic biopsy specimens which had been specially fixed in PLPD, and which were retrieved from the Edinburgh University Pathology Department archives.

2. ras p21 IMMUNOHISTOCHEMICAL STAINING OF CONTROL TISSUES

Y13 259 produced specific membrane and cytoplasmic staining in the positive control breast carcinoma specimen. In sections taken from this specimen, areas of carcinoma showed clear localisation of ras p21 product in the cytoplasm and on the cell membranes of acinar cells of malignant breast lobules. When compared with their non-malignant counterparts, which appeared negative in the same section, the staining in acini composed of malignant cells was significantly enhanced (Fig 14). There was always some degree of weak background staining in the stromal tissue, but this was never of sufficient intensity to obscure the differential staining between the malignant and non-malignant cells.

3. ras p21 IMMUNOHISTOCHEMICAL STAINING OF CERVICAL TISSUES

All cervical biopsies examined displayed strong positive staining, irrespective of the presence of carcinoma, CIN of various grades, koilocytosis, or glandular atypia.

3.1 *SQUAMOUS EPITHELIA*

The normal squamous epithelium showed ubiquitous strongly positive staining, that appeared to show increasing intensity towards the surface, with the basal cells being less intensely stained. All positive staining was observed amidst weaker background staining of the cervical stroma, which seemed impossible to eliminate.

Unlike the situation in the positive control breast carcinoma tissue, there was no correlation between the intensity of staining, and the presence of premalignant or malignant cells in the cervical tissue. In fact, it was the opinion of two independent observers that it was impossible to identify any consistent gradation in the pattern or intensity of staining observed in normal, premalignant, and malignant cervical cells of the squamous lineage.

3.2 *GLANDULAR EPITHELIA*

The pattern of staining observed in the cells of the endocervical glands of the cervix was, however, considerably different. Normal endocervical glands were consistently and unequivocally negative in all 49 cases in which they could be identified, often in the presence of strongly positive normal, CIN or malignant squamous epithelium.

By contrast, though relatively fewer in number, all three cases showing glandular atypia, two of which had associated adenocarcinoma in the vicinity, all displayed ras p21 positivity in cells of glands with atypia or malignancy.

In cases showing benign metaplastic changes in glands, the picture was slightly more variable. Eight out of ten cases showed ras p21 positivity, and included sections displaying

tuboendometrial metaplasia, and all cases showing squamous metaplasia within glands (Fig. 15). In the 2 cases which were negative - one exhibiting endometrial metaplasia, the other immature squamous metaplasia - staining throughout the section was noticeably weak, even in the normal squamous epithelium, suggesting that their non-conformity may be accounted for by technical variation.

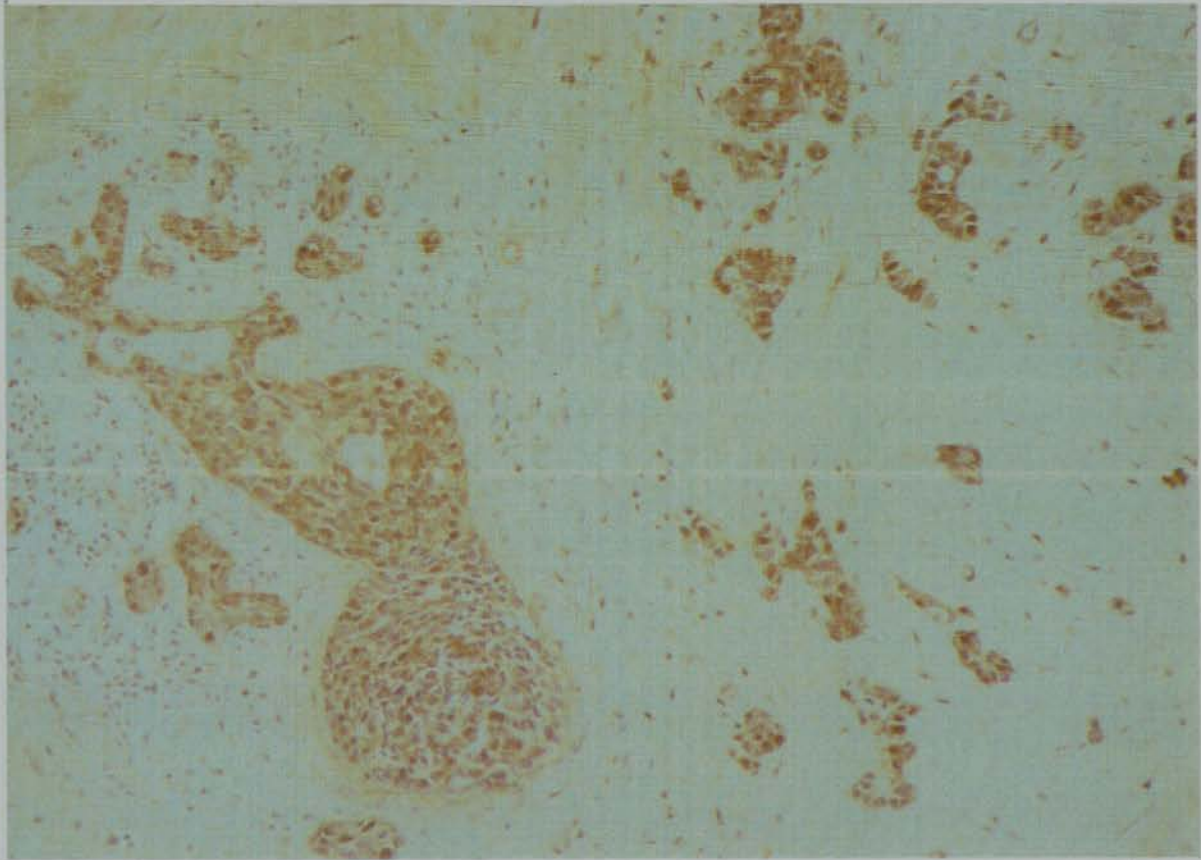


Fig. 14 Immunohistochemical staining with *ras* p21 antibody Y13 259 in a section from the PLPD-fixed positive control breast carcinoma known to express *ras* p21. Strongly positive staining is seen in malignant cells.

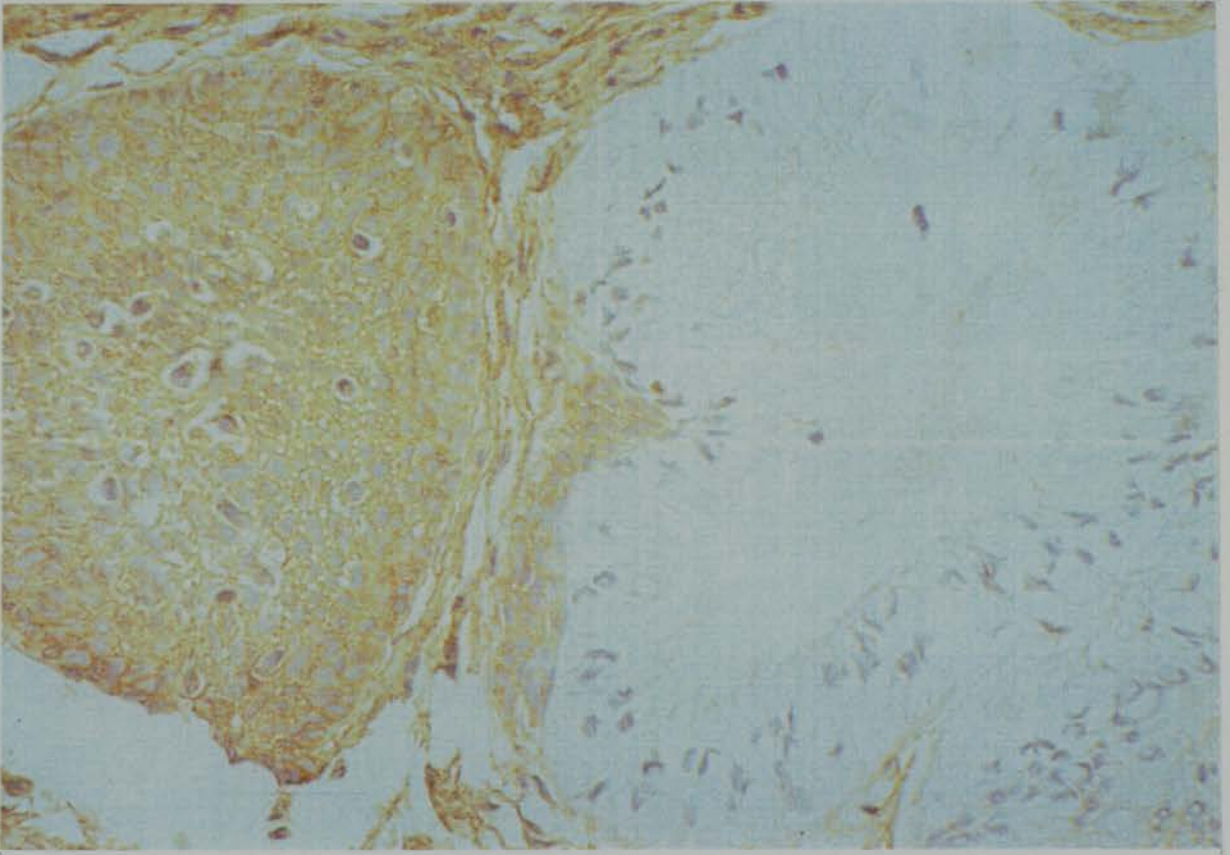


Fig. 15 Immunohistochemical staining with *ras* p21 antibody Y13 259 in a section from PLPD-fixed cervical epithelium. It shows a normal endocervical gland which is unequivocally negative, and an adjacent gland which has undergone squamous metaplasia which is unequivocally positive.

CHAPTER 7

DNA PLOIDY ANALYSIS BY FLOW CYTOMETRY

1. INCIDENCE OF DNA ANEUPLOIDY

There was sufficient frozen tissue available for DNA ploidy analysis by flow cytometry in 52 of the 55 cervical carcinomas which were collected. In the other 3 cases, analysis could not be performed because of unavailability of material.

Two different DNA distribution patterns were observed, and these were classified as predominantly diploid (Fig. 16) or aneuploid (Fig. 17). In some samples, a small diploid peak could be seen on the DNA histogram, in the presence of a distinctly aneuploid peak. In such cases, the aneuploid peak was taken to be representative of the tumour population while the diploid peak was presumed to arise from normal stromal or inflammatory cells in the sample. The mean coefficient of variation (given as half-peak CV) was 3.16% (range 1.13 - 5.86).

The tumour DNA content was centred in the 2C region of the DNA histogram, with small numbers of cells in the 4C ($2 \times 2C$) region in 29 cases. These were classified as diploid tumours, with the small 4C peaks taken as representative of the population of dividing or replicating cells, with a DNA content twice that of the main tumour population (parent cells). In the remaining 23 cases, the DNA value of the tumour population was measured at a distinct peak detected beyond the 2.2C region of the DNA histogram. The average DNA

index of these populations was 1.49. Usually, in these cases a small peak could also be distinguished beyond the 4C region of the DNA histogram, and this was taken to represent the population of aneuploid cells in the replicating phase. The results are summarised in Table XI.

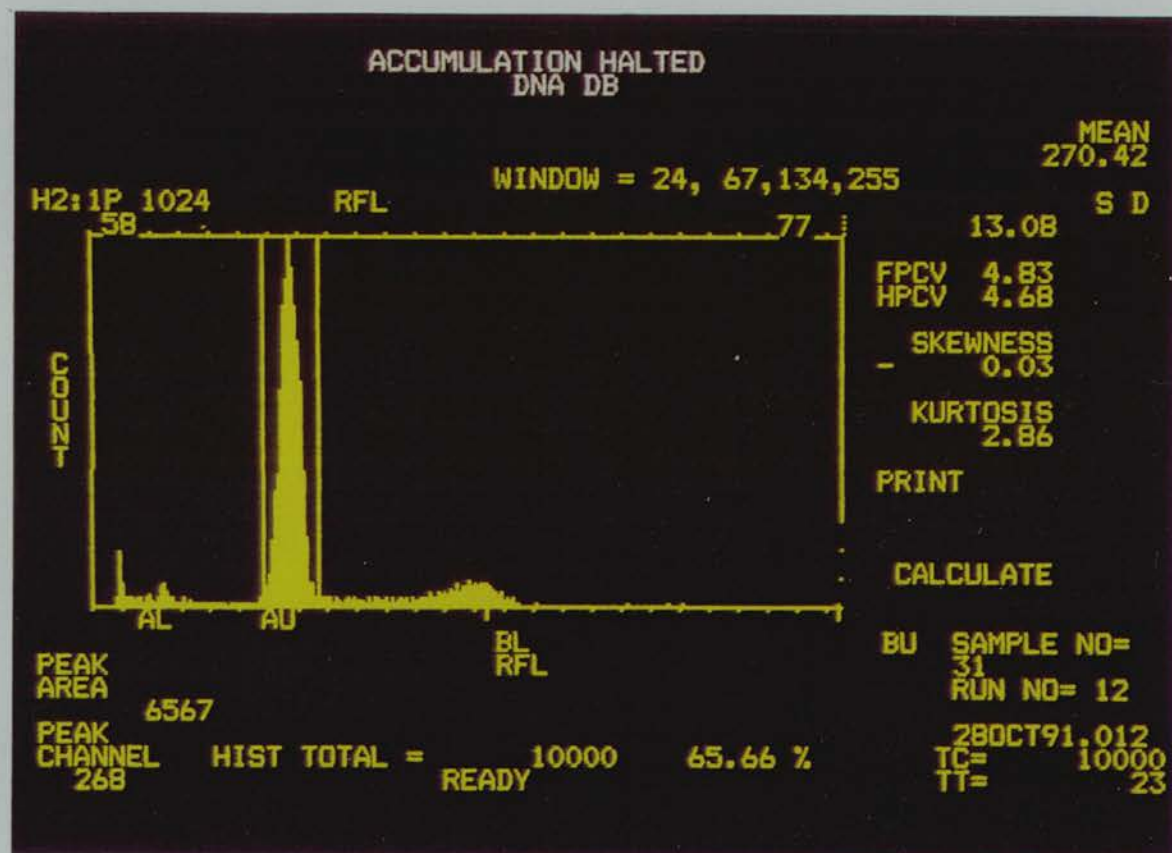


Fig. 16 DNA histogram from DNA ploidy analysis of a cervical carcinoma. This tumour (Case No. 31) shows a diploid pattern.

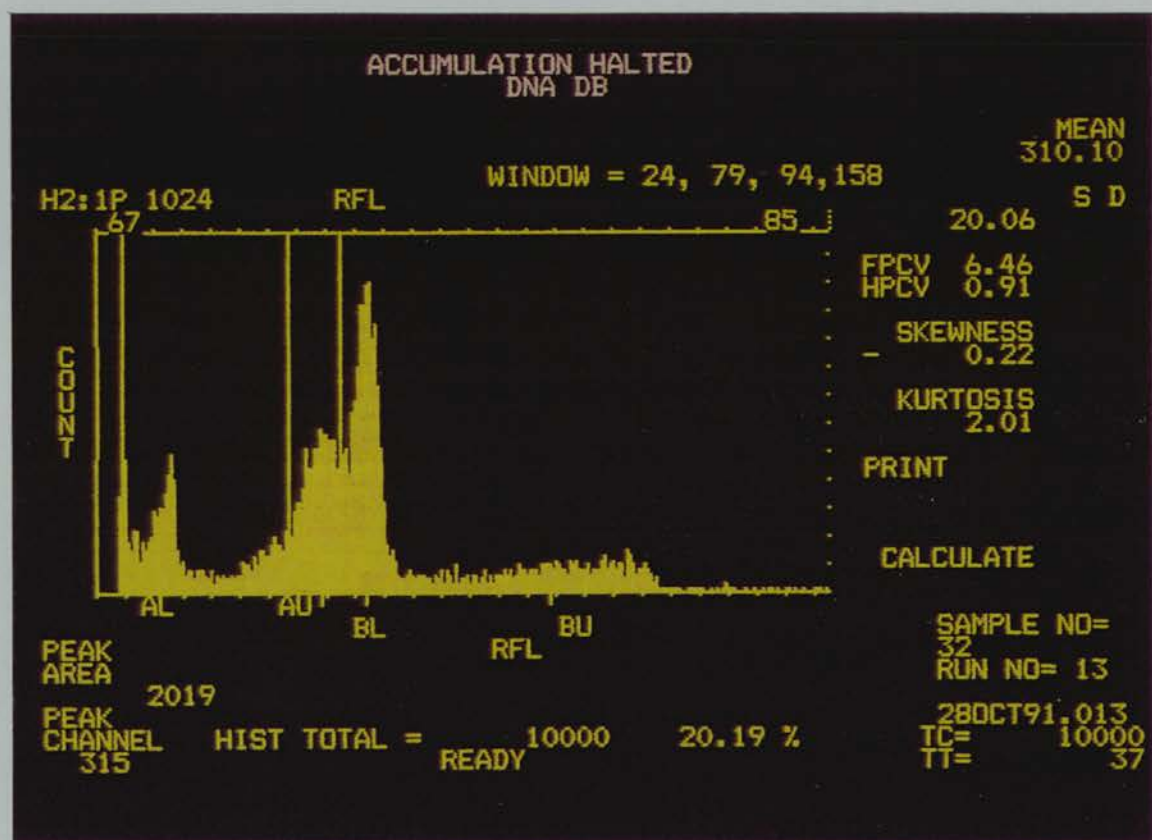


Fig. 17 DNA histogram from DNA ploidy analysis of a cervical carcinoma. This tumour (Case No. 32) shows an aneuploid pattern.

TABLE XI

Clinicopathological Parameters and DNA Ploidy Pattern

CLINICOPATHOLOGICAL PARAMETERS	INCIDENCE OF ANEUPLOIDY (%)	P-VALUE
Clinical (FIGO) Stage		
Early (\leq Stage IIa)	12/30 (40)	NS ^X
Advanced (\geq Stage IIb)	11/22 (50)	
Histological Grade		
Well/moderately differentiated	5/13 (38.5)	NS ^X
Poorly differentiated	8/16 (50)	
Histological Type		
Squamous carcinoma	19/47 (40.4)	NS ^F
Glandular carcinoma	4/5 (80)	
Patient Age		
≤ 35 years	3/11 (27.3)	<0.001 ^S
> 35 years	20/41 (48.8)	
Mean age: Aneuploid 53.4; Diploid 44.3		
Allele Loss Status By RFLP Analysis		
Losses detected	2/8 (25)	NS ^F
No losses detected	4/11 (36.4)	
Human papilloma virus Status		
Positive	17/38 (44.7)	NS ^X
Negative	5/14 (35.7)	
Patient Outcome		
Alive and well	13/28 (46.4)	NS ^F
Ill or deceased	10/24 (41.7)	

Total no. of cervical carcinomas examined for ploidy status = 52

Total no. of aneuploid cervical carcinomas detected = 23 (44.2%)

Total no. of diploid cervical carcinomas detected = 29 (55.8%)

NS = not significant at ≤ 0.05 level S = Student's t-test;

F = Fisher's exact test; X = Chi-squared test

It can be seen that aneuploidy was detected in cervical tumours of all histological types and grades; in tumours from patients of all age groups; and in tumours of all clinical stages irrespective of their HPV, allele loss or mutated p53 status.

2. DNA PLOIDY AND CLINICO-PATHOLOGICAL PARAMETERS

When tumours were grouped according to clinical (FIGO) stage (Table XI), 12 (40%) of 30 early stage (\leq stage IIa) carcinomas were aneuploid, compared to 11 (50%) of 22 of the carcinomas of more advanced stage (\geq stage IIb).

Amongst the 31 tumours for which the degree of histological differentiation had been recorded, there were 29 that had had DNA ploidy analysed. Of these 29 carcinomas, 5 of 13 (38.5%) moderately-well differentiated tumours were aneuploid; while 8 of 16 (50%) poorly differentiated carcinomas were classified as aneuploid.

Aneuploidy was assigned to 19 of 47 (40.4%) squamous carcinomas, and 4 of 5 (80%) glandular (3 adeno-; 2 adenosquamous) carcinomas. Only one of the adenosquamous carcinomas displayed a diploid pattern on the DNA histogram.

Eleven of the tumours analysed had been obtained from patients of 35 years of age or less. Three of these 11 patients (27.3%) were found to have aneuploid tumours, compared to 20 of their 41 counterparts (48.8%) in the > 35 years age-group. The mean age of patients with aneuploid tumours was 53.4 years while that for the diploid tumours was 44.3 years, a difference that was statistically significant ($p < 0.001$; Student's t-test).

Of the 9 tumours in which allele loss(es) had been detected by RFLP analysis, 8 were analysed for DNA ploidy. Two of these 8 tumours (25%) were found to be aneuploid. One (Case no. 4) had shown loss of heterozygosity at loci on 2 chromosomal arms; while the other (Case No. 13) had shown losses at loci on 4 chromosomal arms.

17 of 38 (44.7%) HPV-positive tumours were aneuploid, compared to 5 aneuploid tumours among 14 HPV-negative tumours (35.7%).

Amongst the three tumours showing p53 mutations, there was sufficient material for DNA ploidy analysis in only two. These two tumours (Case Nos. 52 and 53) were both found to have predominantly aneuploid populations on flow cytometric analysis. However, the other 21 aneuploid tumours were found amongst cases in which no mutations could be detected in the hot-spot regions of p53.

Patient outcome was graded 1-4 according to the following criteria:

1. Alive without recurrence after primary treatment
2. Alive with recurrence after primary treatment
3. Deceased from disease
4. Deceased from other cause

The follow up time for these cases ranged from 10 - 29 months, with 95% of cases falling into the follow up period of 12 months or more. Using the criteria above, patient outcome was ascertained for all 55 cases. Of these, 29, 5, and 21 fell into categories 1, 2 and 3 respectively, with no cases in category 4. Table XII shows survival status in relation to

DNA ploidy of tumours.

TABLE XII

Survival status and tumour DNA ploidy

Survival Category	Aneuploid (%)	Diploid (%)
1. Alive and well	13 (25%)	15 (29%)
2. Alive with recurrence	2 (4%)	3 (6%)
3. Deceased from disease	8 (15%)	11 (21%)

Among the correlations between aneuploid status and other clinical and pathological parameters measured (detailed in Table XI), the only one that reached statistical significance was the correlation with patient age ($P<0.001$; Student's t-test). Aneuploid tumours occurred in patients of a significantly older age group than diploid tumours.

PART IV

DISCUSSION

PART IV DISCUSSION

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2. Interpretation of findings
3. Conclusions

CHAPTER 2 DNA FINGERPRINTING

1. Potential for the technique in tumour analysis
2. Technical considerations
3. Interpretation
4. Conclusions

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1. Methodological considerations
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CONCLUDING REMARKS

CHAPTER 1

ALLELE LOSS ANALYSIS

1. METHODOLOGICAL CONSIDERATIONS

RFLP analysis has made an enormous contribution to current knowledge of tumour suppressor genes (TSGs) through its ability to detect allele loss in tumour DNA compared to constitutional DNA of the same individual. In family cancer syndromes, it has been used in conjunction with pedigree analysis to examine DNA from generations of families, using polymorphic markers in chromosomal regions of interest, to map more closely regions of the genome implicated by cytogenetic analysis. In sporadic cancers, this technique has also been applied to the analysis of sporadic genetic events which may contribute to the development of these tumours. The power of RFLP analysis in investigating TSGs lies in part in its ability to be applied to virtually all neoplasms (Benz, 1990), and because of this, much recent work has focussed on a diverse array of sporadic human cancers.

In sporadic cancers, the use of highly polymorphic probes permits comparison of tumour-derived DNA with constitutional (presumed normal) DNA from the same individual, under the same analytical conditions. Sub-microscopic alterations in DNA can be detected that are not always cytogenetically demonstrable. In particular, allele loss can be detected as loss of heterozygosity (LOH), and the consistent observation of LOH at a specific locus from a particular tumour type can act as a guide to the location of putative

TSGs involved in its genesis. Indeed, the use of LOH analysis in identifying the location of candidate TSGs, has now become an established and accepted technique which has led to the isolation of and cloning of some TSGs including Rb-1, p53 and DCC tumour suppressor genes (Friend et al., 1986; Oren, 1985; Fearon et al., 1990).

Nevertheless, the application of LOH analysis to the identification of TSGs involved in sporadic tumorigenesis is not without practical problems and limitations. A major difficulty arising in analysis of sporadic cancers for which no familial form has been identified, and which have not been associated with any inherited syndrome, has been the identification of chromosomes or chromosomal regions "of interest" on which to focus analysis. The current availability of over 2,000 RFLP markers covering all the human chromosomes (Sager, 1989) made the choice of focus even more difficult in the absence of markers for specific chromosomal regions of interest. Newer techniques of comparative genomic hybridisation and chromosomal "painting" may solve these problems, but such techniques are only now becoming available. Other problems that arise are perhaps intrinsic to the technique itself, and its limitations, rather than to the specific tumour type being analysed.

Firstly, LOH can only be detected in patients exhibiting constitutional heterozygosity at the locus of interest. This necessarily restricts the data generated from any tumour population series to heterozygous cases, as homozygous cases are uninformative. This limitation can be overcome to some extent by the use of highly polymorphic probes which hybridise to regions of the genome which, in any given population, exhibit a high level of heterozygosity.

Secondly, the presence of normal stromal and/or inflammatory cells in even the most meticulously dissected tumour tissue specimens, may confound the LOH incidence detected. In heterozygous cases, the presence of contaminating normal cellular DNA may obscure allele loss in tumour cell DNA, so that some cases may go undetected. In this study, it was attempted to minimise contamination by careful dissection of tumour samples from normal cervical tissue prior to DNA extraction.

Finally, while LOH analysis can identify loss of an allele at a particular chromosomal location, it can tell nothing of the mechanism by which that loss has occurred, or of its consequences. For example, detection of LOH is unable to give any indication of the effect, if any, on tumour cell phenotype. By the same token, the detection of LOH in the absence of pedigree analysis (often of no relevance) in sporadic cancers, gives no information with respect to the remaining (undeleted) allele. Indeed, while the presence of a germ-line mutation can be interpreted or predicted from pedigree analysis in family cancer syndromes, in the absence of such data, the presence of mutation in the remaining allele has either to be assumed or determined in each case by other techniques such as DNA sequencing. LOH analysis is therefore unable to detect TSG inactivation resulting from non-deletional events such as mutation.

It thus becomes clear that while it is sufficient in sporadic cancers to compare constitutional and tumour DNA to detect LOH in chromosomal regions of interest, it serves only as an initial guide to the location of potential candidate TSGs, and thereby highlights regions of the genome upon which more detailed mapping and sequencing exercises can be focussed. Given its limitations, it has nonetheless revealed that LOH in the vicinity of TSG loci is a frequent occurrence in a variety of human cancers. Further,

the LOH data generated has led to the identification, location, isolation, and in a few cases, cloning of putative TSGs. So far, its application to cervical carcinoma has been limited.

Cervical carcinoma, with its identifiable premalignant phases, is perhaps an ideal malignancy in which to analyse genetic events such as allele loss, as lesions identified in tumour samples can be analysed specifically in the preinvasive phases, in an attempt to pinpoint critical genetic events in cervical carcinogenesis. On the other hand, cervical carcinoma is a sporadic cancer for which no familial form has yet been identified, and which so far has not been associated with any inherited syndrome. Few clues therefore exist to the location of candidate TSGs. The small tumour size, particularly in the developed world, where diagnosis is usually made in the early stages of disease, severely limits tumour tissue (and hence DNA) availability in individual cases. Further, a dense inflammatory infiltrate is common in these tumours, and this together with the presence of admixed normal stroma throughout the malignant tissue, poses problems in interpretation of LOH data.

2. INTERPRETATION OF FINDINGS

Most available reports on LOH in cervical cancer have therefore been from small series with analysis restricted to specific, and perhaps randomly selected, regions of the genome. This study too has analysed a relatively small series of cases of cervical carcinoma, and has searched for LOH with 22 probes in regions on 8 chromosomal arms, some of which had not been analysed previously in this tumour type, and perhaps adds

another piece to the large cervical carcinogenesis jigsaw.

The 22 probes used in RFLP analysis of this series of 20 cervical carcinomas were highly polymorphic, and hybridised in the vicinity of known oncosuppressor gene loci. With the exception of the locus defined by the marker on chromosome 8q (not as yet identified as a tumour suppressor gene site, and here used as an "innocent" or "control" site), all the regions examined in this series had previously been shown to display high frequencies of deletion or allele loss in several other common solid tumours. The results obtained therefore permitted a comparison of the level of allele loss seen in this series of cervical carcinomas at specific TSG loci, with that observed at the same sites in other common solid tumours.

An important finding of this study of cervical carcinomas was the low incidence of LOH at the known oncosuppressor sites, which contrasts with the higher frequencies reported in other major solid tumours (Table XIII). Although only a limited subset of chromosomal regions was examined, the results suggest that these oncosuppressor genes, commonly implicated in other human tumours, do not play a significant role in cervical carcinogenesis.

2.1 CHROMOSOME 17

Allele deletions on chromosome 17p have been reported in up to 61% of breast carcinomas (Mackay et al., 1988a); 73.1% of osteosarcomas (Toguchida et al., 1989); 75% of colonic carcinomas (Vogelstein et al., 1988); 50-60% of epithelial ovarian carcinomas (Eccles et al., 1990; Russell et al., 1990); up to 55% of brain tumours (Fults et al., 1989) and 63% of bladder carcinomas (Tsai et al., 1990) - suggesting the presence

of a tumour suppressor gene that is involved in a carcinogenic mechanism common to all of these tumours. Data from this study show only 15% of informative tumours with 17p allele loss; a similar proportion to that identified in a recently published series of cervical carcinomas (Kaelbling et al., 1992), but a significantly lower proportion ($P < 0.05$; Fisher's Exact Test) than that observed using similar probes in tumours of breast, bladder, ovary, bone, and colon. This may imply that any association between 17p allele loss and carcinogenesis does not extend to cervical carcinoma.

A potential TSG located in the region of chromosome 17q defined by the VNTR probe pTHH59 has been implicated in ovarian carcinogenesis, and high levels of allele loss have been detected, using this marker. Of 13 heterozygous cases, Eccles et al reported LOH with pTHH59 in 10 cases (77%) (Eccles et al., 1990). By comparison, the frequency of LOH observed with pTHH59 in this series of cervical carcinomas was significantly lower ($p < 0.001$ - Fisher's Exact Test), with no losses observed amongst 13 cases showing constitutional heterozygosity.

2.2 CHROMOSOME 13q

An equally low frequency of LOH was observed within the Rb gene in this series of cervical carcinomas. The Rb gene has been implicated, not only in retinoblastomas, but also in many other more common solid tumours. In particular, allele losses within the Rb gene have been detected in 38% of breast carcinomas (Devilee et al., 1989) and 43% of osteosarcomas (Toguchida et al., 1988). Using the same probe, p68RS2.0, a VNTR probe which hybridises to a sequence within the Rb gene, no allele losses were detected among 14 informative cervical carcinoma cases. This result is statistically significant when compared to those in breast and bone cancers ($p < 0.001$ - Fisher's Exact Test).

2.3 CHROMOSOME 11

Research on cervical carcinoma cell lines has suggested a role for genes on chromosome 11. Microcell transfer of a single copy of fibroblast chromosome 11 into tumorigenic HeLa cells converted them into a non-tumorigenic state (Saxon et al., 1986); and a putative tumour suppressor gene identified in HeLa cells has been mapped to the chromosome 11q.13 region (Srivatsan et al., 1991). Loss of heterozygosity on chromosome 11, in 30% of cervical carcinoma cases, has been reported in a recent study (Srivatsan et al., 1991); while 36% LOH on chromosome 11p had been reported in a previous series (Riou et al., 1988a).

Analysis in this series, using four markers on the short arm of chromosome 11, revealed a frequency of LOH lower than that observed with equivalent probes in breast cancer (Mackay et al., 1988b); and, for three of the four probes used, the incidence of LOH was lower than that observed at the "innocent" locus on 8q. It therefore appears unlikely that an oncosuppressor gene of major importance to cervical carcinogenesis resides on the short arm of chromosome 11. An analysis of the long arm of this chromosome was not performed because of unavailability of DNA probes, but analysis of the frequency of LOH on chromosome 11q in cervical carcinoma / blood DNA pairs may provide a route for further investigation in the future.

2.4 OTHER CHROMOSOMAL ARMS

Losses on chromosome 5q in the region of the APC and MCC tumour suppressor genes, and on 18q in the vicinity of the DCC gene have been associated with colorectal cancer at levels of 41.5% (Ashton-Rickardt et al., 1991), and 71.0% (Fearon et al., 1990)

respectively. In this series of cervical carcinomas, LOH was not detected at high frequency at these loci.

Previously published work reported the occurrence of allele losses in all of 9 informative cases on chromosome 3 at p14-21 (Yokota et al., 1989). Although the results of this series do not directly refute their findings, as the probe used by Yokota et al., was not available, no significant LOH was demonstrated at a locus just bordering on this region (3p.21).

3. CONCLUSIONS

As no region was identified as consistently lost in this series of cervical carcinomas, it was not considered appropriate to extend analysis to CIN lesions as had been intended.

This is a relatively small series of cervical carcinoma cases, but even taking this into account, the lower frequency of allele loss is statistically significant for most of the regions analysed when compared to the results of similar analyses in other tumour types. This suggests that if oncosuppressor genes are involved in cervical carcinogenesis, they are probably found at loci different from those commonly deleted in other solid tumours, and implies that future studies in pursuit of TSGs involved in carcinoma of the cervix should focus on different genetic loci from those so far commonly implicated and investigated in this series. Alternatively, it may be that some other mode of carcinogenesis, perhaps involving HPV or other virus types, but which does not involve TSG inactivation is important in cervical carcinogenesis.

TABLE XIII

COMPARATIVE LOSS OF HETEROZYGOSITY

Published Series of Other Tumour Types vs.This Series of Cervical Carcinomas

Published Series - Other tumour types					This series -Cervical carcinoma		
Chr	Tumour Type	Reference	A/B	%	A/B*	%	p
17p	Breast	Devilee et al., 1989	30/49	61	1/12	8	.001
	Bladder	Tsai et al., 1990	15/24	63	1/12	8	.004
	Ovary	Eccles et al., 1992	10/18	56	1/9	11	.042
	Bone	Toguchida et al., 1989	19/26	73	3/20	15	<.001
	Colon	Vogelstein et al, 1988	45/60	75	3/20	15	<.001
17q	Ovary	Eccles et al, 1990	10/13	77	0/13	0	<.001
13q	Breast	Devilee et al, 1989	12/32	38	0/14	0	<.001
	Bone	Toguchida et al, 1988	13/30	43	0/14	0	<.001
11p	Breast	Mackay et al, 1988b	4/7	57	1/14	7	.025
	Bladder	Tsai et al, 1990	9/23	39	1/15	7	NS
5q	Colon	Ashton-Rickardt et al, 1991	44/106	42	5/16	31	NS
3p	Lung	Naylor et al, 1987	9/9	100	1/4	25	.014
	Kidney	Zbar et al, 1987	11/11	100	1/4	25	.009
18q	Colon	Fearon et al, 1990	29/41	71	1/5	20	.018

CHR.= Chromosome

A = No. of cases with loss of heterozygosity

B = No. of informative cases

% = Percentage of informative cases with loss of heterozygosity

* = Same probe/s used in this series as in published series compared

P = P-value (Fisher's Exact Test)

NS = Not statistically significant

CHAPTER 2

DNA FINGERPRINTING

1. POTENTIAL OF THE TECHNIQUE IN TUMOUR ANALYSIS

Since the discovery of the minisatellite probes 33.15 and 33.6 (Jeffreys et al., 1985a & b), DNA fingerprinting has found application in a wide variety of clinical and non-clinical settings, including forensic science, human identification, paternity verification and pedigree analysis. The belief that cancer is a multistep process resulting from an accumulation of somatic changes in tumour DNA, coupled with the usefulness of DNA fingerprint analysis in identifying somatic differences in DNA, suggested this as a novel approach for studying somatic changes in tumour DNA (Thein et al., 1987).

Allele loss or deletion, or chromosomal loss leading to loss of associated minisatellite fragments are only some of the possible mechanisms by which DNA fingerprint alterations could occur in tumours. It has been suggested that localised amplification of DNA including a minisatellite could result in specific band intensification, and previous studies have identified novel fragments in tumour DNA fingerprints presumed to be the consequence of changes in length of pre-existing minisatellites resulting from unequal sister chromatid exchange (Thein et al., 1987).

Given the comparatively low frequency of allele loss observed in the 20 cervical carcinomas on 8 chromosomal arms examined, the high frequency of allele loss reported in

carcinomas of breast, ovary and colon, the capacity of DNA fingerprinting to screen simultaneously a large number of loci for genetic alterations, and the ease of access to tumour/blood DNA pairs from colleagues with interests in LOH in non-cervical carcinoma, DNA fingerprinting was considered to be a useful method for comparing the frequency of somatic changes in cervical and non-cervical carcinomas. It was anticipated that the level of allele loss may have been reflected in the incidence of tumour DNA fingerprint alterations. If a level of alterations similar to that in other tumours was found, it might have suggested that similar mechanisms of carcinogenesis were operating, but involving other tumour suppressor gene loci. Conversely, if the incidence of DNA fingerprint alterations in cervical carcinomas was lower than that in other tumour types, it might have implied a different mechanism of carcinogenesis to be involved.

2. TECHNICAL CONSIDERATIONS

To this end, minisatellite probes 33.15 and 33.6 were used with the novel repeat unit multipriming (RUMP) and hybridisation method (Ferrie et al., 1991). This method is believed to improve labelling and hybridisation of these probes, and to result in a more rapid, efficient and reproducible technique than was hitherto available. The use of $\alpha^{32}\text{P}$ -dGTP as opposed to conventional $\alpha^{32}\text{P}$ -dCTP with this method produced "hotter" and better fingerprints; and interpretable fingerprints were generated within a shorter (48 hour) period. The main advantages of this system lie in its requirement for relatively small quantities of DNA (1-2 μg) compared with LOH analysis; its ability in theory to screen up to an estimated 50 loci (if both probes are used); and its applicability to DNA extracted from any tissue type.

A drawback of the DNA fingerprint technique is that, as the minisatellites detected are dispersed throughout the genome, no clues are provided by the fingerprint as to the chromosomal location of any lesions identified. As with all analyses involving DNA, errors in data interpretation may result from sample contamination eg. contamination of tumour DNA by normal DNA from stromal and/or inflammatory cells, or cross contamination of samples resulting from poor technique. Scrupulous attention to sampling technique can minimise these errors.

More specific problems may arise from incomplete digestion of DNA which commonly occurs with Hinf I, the restriction endonuclease used in these reactions. In an attempt to overcome this, a relatively high concentration (80u/μl) of enzyme was used in order to limit the inhibitory effects of glycerol (used 50:50 to make up the enzyme solution), and digestion was carried out at 37°C for a minimum of 16 hours. As an adjunct, completeness of digestion was confirmed with the use of the single locus probe MS51 which showed two predominant bands when DNA digestion was complete, but more than two bands when it was incomplete.

3. INTERPRETATION

In keeping with previous reports, up to 15 hypervariable fragments could be interpreted with the probe 33.15 and up to 11 bands with probe 33.6 (Jeffreys et al, 1985b). However, in all cases, the smaller hypervariable segments merged with one another and resulted in an uninterpretable smudge of bands in one half of the fingerprint.

As previously reported in another series of diverse cancers (Thein et al., 1987), the majority of cases in this series (15/23 - 65.2%) showed no alteration in the tumour DNA fingerprint when compared to the corresponding constitutional DNA fingerprint. 8 of 23 cases (34.8%) in this study showed DNA fingerprint alterations compared to 10 of 35 cases (28.6%) in a previously reported study (Thein et al., 1987). The same types of alterations were observed, namely deletion or loss of hypervariable segments, increased or diminished intensity of bands, and the acquisition of novel hypervariable fragments. Deletion or loss of fragments was the most common alteration observed.

It was interesting to note that taking the results of both probes 33.15 and 33.6 together, only 2 of 10 cervical carcinomas (20%) showed alterations in their tumour DNA fingerprint; while 7 of 15 (47%) of those of non-cervical origin exhibited alterations. The numbers are perhaps too small for meaningful conclusions to be drawn, but the pattern suggests a higher frequency of detectable alterations amongst breast, ovarian and colonic carcinomas than cervical carcinomas.

In one cervical carcinoma that exhibited high levels of allele loss (LOH detected at 7 sites by RFLP analysis), the tumour and constitutional DNA fingerprints were identical with both probes. This perhaps highlights the fact that although DNA fingerprints obtained from minisatellite probe 33.15 are derived from an estimated 30 loci (Jeffreys et al., 1986), the probe does not detect alterations over large parts of the human genome.

4. CONCLUSIONS

These results suggest that while it is claimed that DNA fingerprinting using probes 33.15 and 33.6 is sufficient in the vast majority of cases for human identification, parental verification and authentication of human cell lines, and may offer another approach to the detection of somatic alterations in tumours, it is insufficient to identify or characterise all of the subtle and site-specific alterations in the genome which occur in the process of carcinogenesis. Given the small number of tumours examined, it would be premature from these results to deduce that the frequency of somatic alterations is lower in cervical than in other common solid tumours.

CHAPTER 3

HPV ANALYSIS

1. METHODOLOGICAL CONSIDERATIONS

Several methods are currently available for the detection of human papillomavirus (HPV) DNA in tissues and in DNA extracted from them. These have been discussed at length in Chapter 3 Section 3 of the Introduction. Polymerase chain reaction offers several distinct advantages over other methods, firstly in specificity - type specific detection is possible; and secondly in sensitivity - only a small amount of material is required for detection (theoretically one HPV genome; in practice 500 ng samples as used in this study).

With over 60 types of HPV identified, it is a formidable task to attempt to screen any series of cases for all the known types and subtypes. In this study, investigation was therefore initially (first 20 carcinomas examined) restricted to the 5 HPV types most commonly associated with genital lesions, namely types 6,11,16,18 and 33. Thereafter, for reasons previously alluded to in Chapter 1 of the Results, HPV screening by PCR in the latter 35 carcinomas was further restricted to types 16,18 and 33 - the types most commonly associated with human genital cancer.

PCR primer pairs specific for HPV types 6,11,16,18 and 33 were employed. The specificity of each primer pair had previously been demonstrated (Arends et al., 1991) by their ability to detect and amplify sequences specific to the HPV plasmid type for which they were

designed, and their inability to do this with plasmid DNA of the other 4 HPV types under investigation. The sensitivity of the technique was demonstrated by the ability of each primer pair to successfully detect and amplify the appropriate HPV DNA sequence from a PCR reaction concentration of HPV plasmid DNA as low as 0.1ng/100µl of reaction.

Positive controls using these type-specific primers with their respective HPV plasmid DNA as template were included in each run to confirm that specific detection occurred. Negative controls in which sterile water was substituted for template DNA excluded the possibility of false positives due to primer dimer formation or contamination of non-genomic reagents.

Two methods were used to minimise the occurrence of false negative results. Firstly, experiments were repeated to confirm initial findings. Secondly, ras primers were used for detection and amplification of ras sequences (ubiquitously present in all human tissue specimens) to confirm that sufficient template DNA had been used in all reactions. Separate storage of PCR reagents, HPV plasmid DNA, and PCR products; the use of personalised positive displacement pipettes; and attention to detail in setting up PCR reactions and incubations were all used in an attempt to minimise contamination and hence false positive data.

2. INTERPRETATION OF FINDINGS

2.1 PREVALENCE OF HPV TYPES

Human papilloma viruses, especially types 16 and 18, have long been associated with

malignancy in the cervix (Bosch et al., 1989; zur Hausen, 1989a; Chang, 1990; Singer & Jenkins, 1991). Having used the PCR technique and adhered to the above mentioned criteria, the results of HPV screening in this series of cervical carcinomas were in keeping with existing data on the prevalence of these 5 HPV types.

It is interesting that neither types 6 nor 11 could be detected in any of the first 20 invasive cervical carcinomas examined. Previous work has shown these types to be more commonly, though not necessarily exclusively, associated with benign genital lesions such as condylomata rather than invasive carcinoma of the cervix.

All 55 carcinomas were examined for types 16, 18 and 33. In keeping with the current literature, HPV type 16 was the type detected most frequently, and was present in 28/55 (51%) of invasive carcinomas; 28 of 39 (72%) of HPV positive carcinomas; and 28 of 49 (57%) of invasive squamous carcinomas.

HPV 18 was detected less frequently than HPV 16, though significantly more frequently than HPV 33. HPV 18 was detected in 20 of 55 (36%) of invasive carcinomas; 20 of 39 (51%) of HPV positive tumours; and 20 of 49 (41%) of invasive squamous carcinomas.

It is interesting that both types 16 and 18 were detected in 10 of 55 (18%) of invasive carcinomas; and 10 of 39 (26%) of HPV positive cancers; but that these cases appeared to show no distinguishing features in terms of patient age, tumour type, grade or prognosis.

Over the entire series of 55 cases, HPV 33 was detected in only one case (2%).

2.2 HPV 18 AND ADENOCARCINOMA

In this series, there is perhaps a slightly higher prevalence of HPV 18 positive tumours than is generally quoted in the literature. Indeed, it has previously been suggested that HPV 18 is more commonly associated with the less prevalent invasive adenocarcinoma of the cervix than with the squamous type (Jaworski, 1990).

The percentage of carcinomas of glandular type (adenocarcinoma and adenosquamous carcinoma) in this series, 6 out of 55 (11%) is unfortunately too small for any meaningful conclusions to be drawn with respect to this association. However, amongst the 6 glandular carcinomas examined, it is interesting that HPV 18 DNA was detected in all but one of them (83%) - the remaining carcinoma being negative for all the HPV types examined. On the other hand, HPV 18 DNA, in the absence of HPV 16 DNA was detected in only 7 of 33 (21%) HPV positive squamous carcinomas.

Although there is only a small number of glandular carcinomas in this series, the results lend support to the proposal of an association between HPV 18 and adenocarcinoma. However, a larger series of cases is required to substantiate this claim.

2.3. INTEGRATED VERSUS EPISOMAL STATUS

Previous studies have suggested that HPV DNA tends to be integrated into the host cell genome more frequently in malignant lesions, while it is more commonly episomal in benign lesions (Pfister, 1987). The finding in this series that the HPV DNA was integrated in 9 of 12 (75%) HPV-positive tumours analysed is in agreement with this proposal. However, if the virus is regarded as an aetiological agent causing DNA damage in cervical carcinogenesis, it remains difficult to explain the detection in some tumours of viral DNA

in its episomal state only. A "hit and run" mechanism has been proposed for herpes viruses in cervical carcinogenesis. It may be that this also occurs with HPV in some cases. Alternatively, the virus may be an incidental finding, present only as a passenger, and unrelated to aetiology of the cancer in cases in which its DNA does not become integrated into the host cell genome.

With regard to sites of viral integration, there have been a few studies suggesting specific sites of viral integration in relation to the myc oncogene (Durst et al., 1987), and in relation to fragile chromosomal regions. Most of these reports have been based on in vitro studies using cervical carcinoma cell lines. The bulk of available evidence suggests ubiquitous and non-specific sites of integration. This pattern is certainly supported by the findings in this study where, in the first 20 carcinomas examined, neither the presence nor the integration of the HPV types investigated showed any consistent correlation with the allele losses or deletions that were observed on the 8 chromosomal arms investigated by RFLP analysis.

2.4. HPV NEGATIVE CARCINOMAS

Neither HPV 16, 18 nor 33 could be detected in 16 of the 55 cervical carcinomas (29%) analysed by the sensitive PCR technique, despite successful PCR amplification of their ras sequences from equivalent DNA samples. HPV-negative cervical carcinomas have previously been recognised and reported in the literature. Their identification supports the view that HPV infection is neither the only aetiological factor nor a necessary prerequisite for the development of cervical carcinoma.

More recently, the existence of HPV-negative cervical carcinomas has assumed greater importance because of the putative role of mutated p53 tumour suppressor genes in these

cancers. The relationship of p53 and HPV status in this series of cervical carcinomas is discussed in the following Chapter in greater detail.

3. CONCLUSIONS

The association between HPV and cervical carcinoma has been confirmed by PCR in 71% of the cases in the present study. However, the finding that common HPV types 6, 11, 16, 18, and 33 were absent in 29% of the tumours examined suggests that HPV infection may not be a prerequisite for malignant transformation in the cervix.

Both HPV-positive and HPV-negative tumours showed a low frequency of LOH, and there was no consistent relationship between LOH and the integrated or episomal state of the virus. This suggests that cervical carcinoma cells arrive at the malignant phenotype by pathways which are probably different from those identified in other common solid tumours, but which do not necessarily involve the common HPV types.

CHAPTER 4

p53 MUTATIONS

1. IMMUNOHISTOCHEMISTRY

The very low incidence of positive immunohistochemical staining in biopsies from cervical malignancies contrasts with the findings in other tumours (Bartek et al., 1991), but particularly with findings in breast (Cattoretti et al., 1988), lung (Iggo et al., 1990), ovarian (Eccles et al., 1992), colonic (Purdie et al., 1991), and squamous skin carcinomas (L. Stark, personal communication) where overexpression of p53 protein is frequently seen. Furthermore, in breast (Davidoff et al., 1991) and endometrial carcinoma (Kohler et al., 1992), a strong correlation has been found between p53 expression and advanced disease.

In this study, it was found that immunocytochemically detectable levels of overexpression of p53 protein do not occur frequently in cervical carcinoma or in its preinvasive phases. The few positive nuclei detected by immunocytochemistry, in 13 of 115 cervical biopsies (8 of 57 carcinomas), were sparsely distributed in comparison with the positive colorectal cancer controls; occurred in both benign and malignant lesions; and perhaps represented cells in which p53 levels were elevated because of DNA damage, as proposed in the "guardian of the genome" theory. This theory proposes that normal p53 acts as a "molecular policeman" monitoring the integrity of the genome, such that if DNA is damaged, p53 accumulates and switches off replication to allow extra time for its repair (Lane, 1992).

It has been suggested that any p53 protein detected immunohistochemically is likely to be the mutant protein (Lane & Benchimol, 1990). However this has been questioned, and it is believed that every mutation in p53 does not necessarily lead to its accumulation. False positives resulting from non-mutational stabilisation of the protein, and possibly involving interruption to its normal degradative pathway have been identified in cell lines (Wynford-Thomas, 1992).

In this series of 47 cervical carcinomas, where p53 expression was immunohistochemically detectable in only a minority of cases (8/47), the virtual absence of p53 mutations in the 4 "hot spots" (HSRs A-D) has been confirmed by PCR/DGGE.

2. PCR/DGGE DETECTED MUTATIONS

Amongst the 47 invasive cervical carcinomas further analysed by PCR/DGGE, only one somatic mutation was found in the four mutationally active hot-spot regions (A-D) of the p53 gene. This CGC→TGC transition at codon 175 was the only somatic mutation detected amongst 11 HPV-negative carcinomas, and resulted in an arginine→cysteine change in the encoded amino acid sequence. Codon 175 is a recognised mutational "hot spot", though CT transition in position 1 has not been reported previously (Caron de Fromental & Soussi, 1992).

In two other cervical carcinomas (one an HPV 16-positive tumour; the other an HPV-negative tumour), mutations detected in the "E" region by PCR/DGGE, were also present

in the corresponding constitutional DNA samples. On sequencing, each of these was confirmed as a silent mutation at codon 213, as both sequences encode the same amino acid, arginine. This has been recorded as a rare polymorphism in human populations (Caron de Fromental & Soussi, 1992).

It is notable that no mutations were detected in any of the five p53 regions (including HSRs A-D) examined in this study, in 9 of the 11 HPV-negative cervical carcinomas.

3. COMPARISON OF IMMUNOHISTOCHEMICAL AND PCR/DGGE FINDINGS

In the single carcinoma in which a somatic mutation in p53 was confirmed, the protein was also detected immunocytochemically with antibody MAb DO-7. No p53 protein was immunocytochemically demonstrable in one of the tumours (the HPV-negative cervical carcinoma) in which a silent mutation was detected at codon 213, suggesting that this was one example of a mutation which did not result in accumulation of the protein product. In the other case (HPV 16-positive), in which the same DNA polymorphism at codon 213 was identified, positive staining was observed with both PAb1801 and MAb DO-7, but its sparse distribution did not differ from that seen in cases without p53 mutation, and probably merely represented cells with DNA damage as previously discussed.

4. LOH ON CHROMOSOME 17p AND p53 MUTATION

In other tumours (eg. breast, colon, lung, and ovary), mutation in one p53 allele has

frequently been associated with loss of the corresponding normal allele (Eccles et al., 1992; Iggo et al., 1990; Baker et al., 1990b; Prosser et al., 1990). In keeping with the relative absence of p53 mutations in this series of cervical carcinomas, the incidence of LOH on chromosome 17p (15%) using 6 RFLP markers in the vicinity of p53 was low, and within the range expected as 'background' for any locus examined. Moreover, in the 3 cases showing allele loss, no mutations were detected in the remaining allele in the four hot spot regions analysed.

The frequency of allele loss on 17p in cervical carcinomas in this series is significantly lower ($P < 0.05$; Fisher's Exact Test) than that in breast (61%) (Mackay et al., 1988a), colon (75%) (Vogelstein et al., 1988), ovary (50-60%) (Eccles et al., 1990; Russell et al., 1990), bladder (63%) (Tsai et al., 1990), brain (55%) (Fults et al., 1989) and bone (73%) (Toguchida et al., 1989). The absence of p53 mutations in the 3 carcinomas in which 17p losses occurred, suggests that either the allele losses have occurred as a consequence of the general genetic instability exhibited by many tumours, or that p53 mutations have occurred in regions of the gene different from those most frequently affected in other tumours.

5. p53 MUTATIONS AND HPV STATUS

An association between the HPV E6 and E7 genes and the p53 (17p) and Rb (13q) genes respectively, has been suggested (Banks et al., 1990; Crook et al., 1991c; Scheffner et al., 1990, 1991). Inactivated p53 protein has been associated with HPV 16 E6 oncoprotein complex formation while the Rb gene cellular protein has been associated with HPV 16 E7

protein.

Much uncertainty exists over the status of p53 in cervical carcinoma. Initial studies on anogenital tumours and cell lines suggested an association between HPV 16/18 negativity and the presence of p53 mutation (Crook et al., 1991a). This appeared to be further substantiated by experiments in which p53 DNA and mRNA, from cervical carcinoma tissue and cell lines respectively, were sequenced, which suggested that loss of gene function, either by mutation, or by complexing of its product to that of the HPV E6 transforming gene, may play a crucial role in cervical carcinogenesis.

In these studies on cervical carcinoma cell lines, and more recently on cervical tumour tissue, wild type p53 mRNA and DNA were sequenced from HPV positive cell lines and tumours respectively, while the mutated form was detected only in those that were HPV negative. This led to the suggestion that alternative and mutually exclusive routes for altering p53 function are adopted in cervical carcinogenesis: mutation, or complex formation with HPV E6 (Crook et al., 1991b; 1992).

The findings of this study are not entirely consistent with this hypothesis, in that despite the fact that 11 of 47 (23%) of the carcinomas were negative by PCR for the 3 HPV types analysed (16,18, & 33), only one showed evidence of a somatic mutation in any of the 4 mutational "hot-spots" of the p53 gene (A-D) which were examined. However, these findings do not exclude the possibility that in HPV-positive cases, the wild type p53 gene may be inactivated by complexing to the HPV E6 gene product.

The findings in this study of HPV-negative cervical carcinomas lacking p53 mutation have

been supported by other more recent studies (Borresen et al., 1992; Fujita et al., 1992). Further, it is interesting to note that of over 350 reported point mutations in p53 which were analysed by Caron de Fromental & Soussi (1992), only 2 were associated with cervical carcinoma - both in cell lines rather than tumour tissue (Scheffner et al., 1991).

Although the model suggested by Crook et al. is an attractive one, the results of this and other studies mentioned above suggest that the issue of p53 aberrations in cervical carcinogenesis is a bit more complicated. There remains debate over the implications or relevance of viral infection, now that virus-negative cervical cancers, lacking mutated p53 have been reported (Borresen et al., 1992; Fujita et al., 1992).

The recent isolation of the human homologue of the mouse oncoprotein, MDM2, and its mapping to chromosome 12q, may help to resolve this issue. Like DNA viruses, it too may complex to and inactivate the tumour suppressor activity of p53. In addition, experiments showing that tumours with MDM2 amplification lack p53 mutations suggest that, like DNA viral oncoproteins, MDM2 may also inactivate p53 in the absence of mutation (Lane, 1992).

6. CONCLUSIONS

In conclusion, unlike other common sporadic human cancers, mutation in the highly conserved regions of the p53 gene, in association with allele losses on chromosome 17p in the vicinity of the p53 gene, is not a frequent occurrence in cervical carcinoma. Furthermore, no correlation has been found between p53 status and the presence or

integration of HPV 16, 18 or 33 in the cases examined. Finally, immunohistochemically detectable levels of p53 (due to the presence of presumed mutant protein) have not been found with significant frequency in preinvasive or invasive phases of cervical carcinoma.

Several crucial p53 issues remain unresolved, but their clarification is essential for a fuller understanding of carcinogenesis.

CHAPTER 5

ras p21 EXPRESSION IN CERVIX

1. THE *ras* FAMILY OF ONCOGENES

Recent advances in molecular biological techniques have made it clear that various cellular oncogenes are closely associated with cellular proliferation, and that their inappropriate expression may be involved both in carcinogenesis and in tumour progression. The *c-ras* gene family is commonly implicated in many different malignancies in man (Bos, 1988), and therefore some involvement in cervical carcinogenesis might be anticipated.

The *ras* family of proto-oncogenes includes at least three functional loci in mammals. The *ras* oncogene products of all three genes (*Ha-ras*, *Ki-ras*, and *N-ras*) are 21kD proteins which seem to be localised at the cell membrane, and engage in GTP- binding and GTPase enzyme activities. These proteins have been detected in almost every fetal and adult tissue (Furth et al., 1987), but the level of expression has been found to vary significantly among cell types and stages of cellular differentiation. The ubiquitous distribution of p21^{*ras*} and the variation in its expression in different tissues and at different stages of cellular differentiation, has suggested a role for these proteins in cellular proliferation. However, the variation in levels of expression amongst cell types has also implied a role in certain specialised cellular functions. It remains true however that the normal physiological role of *ras* proteins remains largely unknown.

ras expression has been described in normal cervix. The immunohistochemical staining pattern has been described as similar to that seen in other stratified epithelia such as in the oesophagus, with immunoreactive basal and suprabasal cells, with staining most intense in 1-2 layers above the basal cells (Furth et al., 1987).

Each of the proto-oncogenes, *Ha-ras*, *Ki-ras*, and *N-ras*, is susceptible to mutation, and mutations in codons 12, 13 and 61 in any of the three *ras* genes have been shown to result in active transforming genes. Activating mutations in *ras* oncogenes have been detected in a variety of human solid tumours, including carcinomas of colon, stomach, oesophagus, breast, pancreas, lung, and thyroid, and in leukaemias; with up to 90% of pancreatic adenocarcinomas, and 50% of colonic adenocarcinomas having detectable mutations in some series (Bos, 1989). Loss of *ras* alleles has been reported in human tumours, and gene amplification and overexpression, resulting in elevated levels of *ras* gene transcripts, have also been described in human neoplasia (Spandidos, 1985).

2. STUDIES OF *ras* IN CERVICAL CARCINOMA

Studies of *ras* in cervical carcinoma have been few. Many have involved cell lines and other in vitro systems, and the focus has largely been on the incidence of mutation, allele loss, and gene amplification. Results have been, in some respects, conflicting and inconclusive.

Mutated *ras* has been shown in vitro to cooperate with HPV 16 DNA in transforming primary cells (Matlashewski et al., 1988). Transfection of activated *Ha-ras* to HPV 16

immortalised human cervical cells led to transformation of cells that produced malignant squamous carcinomas when transplanted into nude mice (Di Paolo et al., 1989). However, these are artificially contrived situations, and there may be few naturally occurring analogues amongst spontaneously occurring human neoplasms.

Allele loss at the *c-Ha-ras-1* locus on chromosome 11p has been reported in 36% of a series of 76 cervical carcinomas (Riou et al., 1988a). Mutated *ras* alleles were detected in a proportion, albeit a relatively small one (8 out of 76, 11%) of these primary cervical carcinomas, and were detected both amongst carcinomas which had lost *c-Ha-ras-1* alleles and those which had not. 24% of advanced (> Stage II) carcinomas in this study were found to have mutated *ras* compared to only 2% of early (\leq Stage II) carcinomas. Pinion et al. (1991) described significant amplification of *Ha-ras* genes in CIN3 and invasive carcinoma, when compared to normal cervix. Results from both of these studies led to the conclusion that there is an association between *ras* mutation or amplification and poor prognosis or tumour progression.

Conversely, Bos (1988) detected no *ras* mutations in 30 primary cervical carcinomas studied; and Enomoto et al. (1990) found no *Ki-ras* mutational activation in the 4 cervical carcinomas examined in their series of 27 gynaecological malignancies.

Sagae et al. (1989) investigated *ras* p21 overexpression in 170 cervical carcinomas using an immunohistochemical technique, and found that overexpression, which occurred in over 50% of cases, was related to prognosis. This correlation was dependant on the histological tumour type, with *ras* overexpression correlating in large cell keratinising and non-keratinising tumours with a poor prognosis; while its overexpression in small cell tumours

correlated with a better prognosis.

3. TECHNICAL CONSIDERATIONS

3.1 MONOCLONAL ANTIBODIES

ras p21 expression can be identified immunohistochemically with the use of monoclonal antibodies reacting with *ras* oncogene p21 product. Indeed many reports have been presented on *ras* overexpression in various types of cancer, including not only cervix as previously described (Sagae et al., 1989), but also cancers of breast, colon, lung, bladder, prostate, and pancreas. Sagae et al. (1989) used an anti-*ras* p21 monoclonal antibody rp35 to detect *ras* overexpression. This antibody has been described as having a similar specificity to RAP5, which reputedly recognises a common epitope shared with p21s encoded by all of the activated *ras* gene family, but whose specificity is disputed (Robinson et al., 1986). This study used the rat monoclonal antibody Y13 259, whose specificity for p21 is not in doubt.

Y13 259 was raised in rats bearing tumours induced by the Harvey strain of the murine sarcoma virus (Furth et al., 1982). It has previously been shown to immunoprecipitate human p21 *ras* encoded protein species of Ha-, Ki-, and N-*ras* cellular oncogenes (Furth et al., 1987), and is known to react with the products of both the normal and the mutationally activated *ras* gene.

Its specificity has previously been documented in a variety of different reports (Gallick et al., 1985; Mulcahy et al., 1985). Its specificity and sensitivity were confirmed in this study

by the pattern of staining demonstrated in the positive control breast tissue. Tissue sections from this previously reported Ha- *ras* positive breast carcinoma (Going, 1989) exhibited differential staining between normal and malignant tissue, with the latter showing markedly enhanced staining when compared to its normal counterpart.

3.2 *FIXATION*

The major problem with the use of Y13 259 immunohistochemistry had been the lability of its epitope under conditions of strong aldehyde fixation. Formaldehyde fixation, for example, completely abolishes immunoreactivity of paraffin sections of human tissues, and it has been reported that no useful staining is restored after trypsinisation. Periodate-lysine-paraformaldehyde- dichromate (PLPD) fixation (Pollard et al., 1987), used in this study, has been shown to provide good morphological preservation, without significant loss of p21 immunoreactivity, and for this reason, was the preferred fixative in this study (Going et al., 1988). Positive staining in positive control (breast) and test (cervical) tissue sections, with good morphological preservation in this series, confirmed the suitability of this fixative.

4. CELLULAR LOCALISATION OF *ras* p21 PROTEIN

It was in *ras*-transformed cell lines that the staining pattern of *ras* protein has previously been described. Membrane localisation of p21 *ras* distribution was suggested by the staining pattern of frozen section and PLPD-fixed preparations of embedded FHO5T1 cultures (Williams, 1988). In contrast, localisation of stain in PLPD-fixed human tissues has previously been described in the cytoplasm, and was observed at this location in the

PLPD-fixed cervical tissues in this series. Also as previously reported (Williams, 1988), very occasional nuclear staining was also observed.

There are several possible explanations for the cytoplasmic distribution of staining. First, it may be an artefact of tissue sectioning. Secondly, some degree of perioperative ischaemia or hypoxia occurring prior to or during sample collection may result in an alteration in the cellular distribution of p21 prior to tissue fixation. Alternatively the cytoplasmic distribution of staining may be authentic, and represent the consequence of errors in, or derangement of, post-translational modification of p21 that increase cytoplasmic solubility, and hence distribution of the protein. The significance of the nuclear staining which was infrequently observed in the PLPD-fixed cervical tissues remains uncertain. It may reflect genuine p21 distribution, but the previously reported observation that Y13 259 cross reacts in immunoblots with a histone protein (Williams, 1988), suggests that this pattern is more likely to be artefactual.

5. STAINING PATTERNS IN CERVIX

5.1 *SQUAMOUS EPITHELIUM*

The tissue pattern of staining observed in these cervical biopsy specimens, although interesting is also difficult to explain. Contrary to previous observations, *ras* expression could be detected in all cells of the squamous epithelium, with no differences observed in the intensity of staining between cells of the basal, intermediate or superficial layers, or between cases of normal epithelium, CIN of all grades, or invasive squamous carcinoma. This implies that there are no immunohistochemically detectable differences in *ras* p21

expression between normal cervical squamous epithelium, and that of preinvasive or overtly cancerous cervix; the latter irrespective of tumour stage, grade, or histological type.

However, immunohistochemical staining is fundamentally a qualitative technique. Assessment of results in individual cases should therefore ideally use criteria which take account of the presence or absence of specific staining in the section. Nevertheless, there is no doubt that real differences in the intensity of specific staining do occur, and are likely to reflect true differences in the amount of detectable antigen. In such circumstances, a scoring system of absent, faint, or strong staining that is reliable and reproducible, with a high degree of inter- and intra-observer agreement has to be devised to achieve consistent results and facilitate their interpretation. No such scoring system was required in this series as no variations in intensity were observed, and there was inter- and intra-observer agreement between the presence and absence of staining.

5.2 GLANDULAR EPITHELIUM

The principal result of immunohistochemical staining in this series of cervical tissues was the observation of consistently intermediate levels of p21 expression in the squamous epithelium, in stark contrast to its absence in the epithelium of normal endocervical glands. Further, abnormal (atypical, malignant, and metaplastic) endocervical glands were consistently positively stained. This observation has not been previously recorded in the cervix, and its explanation remains uncertain. In other cell types, high levels of *ras* expression have been associated with proliferation.

It may be that higher levels of expression in abnormal endocervical glands, are a marker rather than a cause of the active proliferation of their metaplastic, atypical, or neoplastic cells. In addition, cells of normal endocervical glands may have a longer cell cycle time than their malignant counterparts, or the normal, premalignant, or malignant squamous cells of the cervix. Alternatively, the carcinogenic process in endocervical glands may require that cells achieve a certain altered proliferative potential - a marker of which is increased *ras* expression - which, once achieved, renders them vulnerable to the action of further carcinogenic stimuli, which convert the lesion to preinvasive disease, or invasive adenocarcinoma.

6. CONCLUSIONS

The findings suggest that *ras* expression remains unchanged in the progression from normal, through CIN, to invasive squamous carcinoma, but is elevated in the progression from normal, through AIS to adenocarcinoma of the cervix. However, as there were few cases of adenocarcinoma and its precursors in this series, more cases need to be studied in order to validate this result.

CHAPTER 6

DNA PLOIDY ANALYSIS

1. BACKGROUND

Normal somatic cells have a diploid number of chromosomes ($2n$), where n is the haploid number of chromosomes found in the normal germ cell. Tumour cells frequently show an abnormal chromosomal complement that is often greater than $2n$ (hyperploid), but sometimes less (hypoploid). Such an abnormal chromosomal number is referred to as aneuploidy, and may be reflected as a change in the total DNA content of the cell.

Aneuploidy probably arises by duplication of chromosomes without cell division resulting in tetraploidy, and subsequent loss (or gain) of chromosomes or parts of chromosomes (Nowell, 1976). Normal or reactive tissue is not associated with DNA aneuploidy (Barlogie et al., 1980) and therefore DNA aneuploidy is held to be implicit of neoplasia, and a marker of malignant potential, reflecting the general genetic instability observed in tumours.

2. DNA PLOIDY ANALYSIS BY FLOW CYTOMETRY

2.1 *PRINCIPLES*

The capacity of some dyes such as propidium iodide to bind to DNA in a stoichiometric

manner permits quantification of DNA present in the nucleus. In flow cytometry, large numbers of cells can be analysed and appropriate statistical methods applied to the resulting data, to give information about DNA content and/or cell cycle variables. Briefly the technique involves the examination of a single cell suspension passing a given point by a suitably tuned laser beam. The fluorescence due to a dye bound to nucleic acids is detected as reflected or transmitted light or both by suitably placed detectors and converted into an electronic signal, from which information about the number of cells and their DNA content can be obtained (Quirke & Dyson, 1986; Hall & Levison, 1990). The DNA content or ploidy of a tumour cell population may then be expressed as the DNA index, defined as the ratio between the DNA content of a tumour cell population and that of a normal diploid cell population.

2.2 LIMITATIONS

Although DNA ploidy analysis provides a rapid, objective, precise and highly reproducible measure of DNA content in a large number of cells, this technique can detect only large quantitative variations in cellular DNA content. It is unable to detect qualitative changes (such as chromosomal translocations, loss of one chromosome with duplication of another, gene deletion in association with amplification), which may not alter total cellular DNA content, but which may be of equal if not more importance to tumour cell phenotype. A "diploid" DNA content therefore confirms a euploid quantity of DNA, but says nothing of its chromosomal distribution.

3. ANEUPLOIDY IN HUMAN TUMOURS

The presence of DNA aneuploidy has been described in malignant lesions with variable incidence (Friedlander et al., 1984a). Flow cytometric measurements of DNA content have increasingly been applied to analysis of solid tumours, and one study has reported a 70% incidence of DNA aneuploidy in 399 solid tumours studied (Newton et al., 1988). The cellular DNA content, and the presence of aneuploidy in tumour cells has in some tumour types been shown to provide important diagnostic information, to be of prognostic significance, and to predict the likely response to therapy (Quirke & Dyson, 1986).

The significance of DNA ploidy to other clinicopathological features of tumours appears to vary according to tumour type. DNA aneuploidy has been reported as a predictor of invasive malignant potential in borderline tumours of the ovary (Friedlander et al., 1984b); as correlating with more aggressive course of disease and lower median survival in ovarian carcinoma (Iversen, 1988); as correlating with increasing size in colorectal adenomas (Van den Ingh et al., 1985), and poor prognosis in rectal adenocarcinomas (Quirke et al, 1987); in bladder cancer, as correlating with histological grade and predicting likely invasion and recurrence in early disease (Chin et al., 1985), and correlating with increased radiotherapeutic sensitivity (Wijkström et al., 1984).

In contrast, although the conclusions of different studies have varied considerably, there are reports that patients with DNA aneuploid neuroblastoma, acute lymphoblastic leukaemia and non- Hodgkin's lymphoma have demonstrated significantly better survival rates when treated with chemotherapy compared to their counterparts with diploid tumours (reviewed in Quirke & Dyson, 1986).

4. STUDIES OF DNA PLOIDY IN CERVIX

There are few reports in the literature comparing the incidence of aneuploidy in cervical tumours with the other clinicopathological features such as stage, grade, age at presentation, and survival after primary therapy. Jakobsen (1984a) in a series of 171 patients, reported that a DNA index of greater than 1.5 was a predictor of recurrence, regardless of stage. It had previously been reported that this was also a predictor of the likelihood of regional lymph node metastases in surgically treated patients (Jakobsen, 1984b).

In a series of 300 patients with cervical carcinoma, major differences in radiosensitivity were reported between DNA aneuploid and DNA diploid tumours, as determined by clinical regression, histopathological response and extent of cell death, the DNA aneuploid tumours showing enhanced radiosensitivity (Dyson et al., 1987).

However, a retrospective study using archival paraffin-embedded material was able to detect unequivocal DNA aneuploidy in only 1 out of 64 cervical carcinoma specimens, although a positive skew was seen in histograms of a further 19 cases, which was probably due to aneuploid components (Hendy-Ibbs et al., 1987).

Jakobsen et al., (1983a) reported an increase in DNA aneuploidy with increasing degrees of dysplasia in CIN. In cervical carcinoma, DNA ploidy was reported to be in agreement with the chromosome number distribution (Jakobsen et al., 1983b), but there have been few studies relating DNA ploidy status with allele loss/loss of heterozygosity.

More recent studies have related ploidy status with grades of CIN and HPV status. Kenter et al. (1993) assessed HPV 16 status in tumour tissue of 69 low stage squamous carcinomas of the cervix, in relation to ploidy, grade and prognosis. No difference was found between the mean DNA index of HPV-positive (n=34) and HPV-negative (n=35) cases, with HPV 16 being detected in both DNA diploid and aneuploid tumours. Jarrell et al. (1992) found that neither DNA ploidy nor HPV status correlated with age, stage, treatment modality and 5 year survival, although advanced stage tumours were more commonly aneuploid.

5. DNA PLOIDY RESULTS IN THIS SERIES

5.1 POTENTIAL VALUE OF DNA PLOIDY ANALYSIS

In order that prognosis be assessed and therapy individualised, a detailed knowledge of prognostic factors is helpful. So far, clinical stage of disease, and the histological degree of tumour differentiation have been the most commonly used prognostic criteria. Both of these are subjective assessments, and hence suffer from poor reproducibility and weak predictive power.

DNA ploidy can be measured objectively, and therefore has the potential to provide a reliable and reproducible index of prognosis, if such a correlation is found. In this study, flow cytometric measurements of DNA ploidy were performed in a series of tumours from 52 patients with cervical carcinoma, and correlations were sought with data from other analyses of LOH, HPV and p53 status. The influence of DNA ploidy on prognosis was also prospectively analysed, together with correlations with clinicopathological features of the

tumours, such as patient age, histological grade and FIGO stage.

5.2 TECHNICAL CONSIDERATIONS

Analysis of DNA ploidy in this series of cervical carcinomas suffered from several drawbacks, which may have influenced the results obtained. The limited availability of tumour tissue meant that only single sample analysis could be performed. Previous reports have indicated that heterogeneity of DNA content is a feature of many neoplasms, and suggest that where possible, multiple samples should be taken to define a tumour's DNA ploidy status (Quirke et al., 1987). Such studies have also demonstrated that heterogeneous tumours have a similar prognosis to pure DNA aneuploid tumours. In this series, the true incidence of DNA aneuploidy may be higher than that recorded, as heterogeneity of ploidy may have gone undetected in apparently diploid tumours because of single sample analysis. However, although only single samples were used, given the small size of the cervical carcinomas investigated, the single samples used were probably representative of the entire tumour.

Single cell suspensions are regarded as an essential prerequisite of the technique. Although no effort was spared in attempting to achieve this level of disaggregation by a combination of mincing, syringing, sieving, filtering, and enzymatic disaggregation, the fibrous stroma of tumours of the cervix proved difficult to disrupt, particularly when compared with other tissue types such as lymphoid tissue and colonic carcinoma tissue, whose textures make their disruption easier.

Cervical carcinomas, by the very nature of their anatomical location, usually bear a heavy leucocytic infiltrate, and tumour cells are often disseminated through areas of normal

cervical stroma. Samples were carefully selected and trimmed, so as to include a majority (>70%) of malignant tissue, with the minimum of normal cervical stroma; but it was virtually impossible to exclude the inflammatory cells, so that all cell suspensions will have had a complement of normal leucocytes. To some extent, this is helpful as it allows quantitation to be relative, in that these act as an internal standard, and diploid control. However, this is only provided that their numbers do not vastly exceed the number of diploid tumour cells, as a small aneuploid cell population may be masked in such circumstances. Chicken erythrocytes, used in these analyses, provided a good external standard, as their DNA content is less than that of human diploid cells, and as such, they do not interfere with the diploid peak during measurements.

5.3 INTERPRETATION OF RESULTS

On the basis of single sample flow cytometric analysis, 23 cervical carcinomas were found to be aneuploid, and 29 diploid. The true diploid number may actually be lower, for the reasons alluded to above. In keeping with previous studies, no significant relationship was found between tumour aneuploidy and tumour stage and patient survival (Jarrell et al., 1992), HPV status (Kenter et al., 1993), tumour type, grade or allele loss status.

The only statistically significant correlation which was found was with patient age, in that patients with aneuploid tumours were on average 10 years older than those with diploid tumours. This finding is intriguing, but such an increased incidence of cellular aneuploidy with age may be accounted for by the fact that older patients had tumours of higher stage. 19/27 (70.4%) of patients with advanced tumours (Stage \geq IIb) were older than the median age of 47 years. However even though advanced tumours were found to be more frequently aneuploid than those of Stage \leq IIa, this relationship was not statistically

significant.

Previous studies have shown enhanced radiotherapeutic sensitivity of aneuploid cervical carcinomas (Dyson et al., 1987). Follow up times are relatively short, but this finding was not reflected in the survival rates in this series, as patients with aneuploid tumours \geq Stage II (hence treated with radiotherapy), fared no better than those with diploid tumours of the same stage, with 6/15 (40%) of the aneuploid group alive and well at 10-29 months follow-up, compared with 6/13 (46%) of the diploid group.

6. CONCLUSIONS

Although the numbers are relatively small, it would appear from this study that ploidy is not an important independent prognostic indicator in cervical carcinoma. Other established parameters such as stage, grade, patient age or others not as yet identified, probably exercise greater influence on prognosis in this disease.

It is interesting that the survival figures for this series of carcinomas would forecast a much lower overall 5-year survival rate for cervical carcinoma, than that quoted in the literature. In this series the mortality rate was 38% (21/55) at ≤ 29 months follow up. Of the 34/55 (62%) who were alive at ≤ 29 months, 5/34 (15%) had already developed recurrence; therefore only 29/55 (53%) were alive and well within 29 months of primary treatment.

CONCLUDING REMARKS

Cervical carcinogenesis is a multistep process, but the individual steps remain enigmatic. In attempting to define the molecular genetic alterations, some differences might have been predicted between cervical carcinoma and other common human malignancies, as there is a strong association with infection with human papillomaviruses in this tumour, and no hereditary form has been identified. While the findings of this study are largely negative, they are significant in highlighting contrasts in the molecular genetic changes in cervical carcinoma compared to other tumour types.

Loss of heterozygosity at commonly implicated oncosuppressor loci was not detected at significant frequency. The prevalence of mutations in the "hot spot" regions of the p53 gene, the most frequently identified genetic lesion in human cancers, was negligible in this series. Furthermore, overexpression of p53 was not detected immunohistochemically in the great majority of cervical carcinomas. No consistent alterations in *ras* oncogene expression were identified in CIN or squamous carcinoma. DNA aneuploidy, although identified in a proportion of cervical carcinomas, showed no correlation with any clinicopathological or genetic parameters other than patient age.

There are alternative explanations for these differences from other common cancers - completely different genetic mechanisms may be operating in carcinogenesis, or similar mechanisms may be occurring but at different genetic loci. For example, it is possible that functional alterations of tumour suppressor genes do not occur, but it seems more likely that as yet unidentified TSG loci are involved. Conversely, although it is conceivable that mutations in p53 gene occur outwith the "hot spot" regions to exert a carcinogenic effect,

it seems more likely that mutations in p53 gene are not fundamental to cervical carcinogenesis. The significance of the interaction between p53 and HPV transforming proteins requires further elucidation.

It is unfortunate that no specific genetic lesion could be identified consistently in invasive cervical carcinomas, as this precluded extrapolation to premalignant phases. Like almost no other common human malignancy, carcinoma of the cervix has a well-defined phase of premalignant evolution which is common, accessible to biopsy, and whose clinical behaviour is well known.

The results of this study, though largely negative, may serve to direct attention to other areas of the genome, where it seems likely that abnormalities will be found. The technology is in place and the opportunity now exists to make real advances in our understanding of the fundamental nature of this disease.

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APPENDIX 1

COMPOSITION OF SOLUTIONS

APPENDIX I - COMPOSITION OF SOLUTIONS

ALLELE LOSS ANALYSIS:

Lysis buffer

0.1M Tris HCl, 20mM NaCl, 1mM EDTA, 0.2% SDS.

10T 0.5E buffer

10mM Tris HCl, pH 7.5, 0.5mM EDTA.

Gel loading buffer

30% Sucrose, 0.4% Bromophenol blue.

20 x TAE buffer

484g Tris base, 114.2ml acetic acid, 200ml 0.5M EDTA made up to 5 litres with distilled water.

Denaturing solution

5g NaOH (0.5M), 219.15g NaCl (1.5M) made up to 2.5 litres with distilled water.

Neutralising solution

292.9g NaCl (2M), 394g Tris base (1M) made up to 2.5 litres with distilled water, with HCl added to pH 5.5.

20 x SSC

876.6g NaCl, 441.2g Na₃ citrate made up to 5 litres with distilled water.

Quick-Hyb buffer

0.5g BSA, 0.5g Polyvinyl pyrrolidone, 0.5g Ficoll, 1g SDS, 1g Na pyrophosphate, 250ml 20 x SSC, 10ml 10mg/ml denatured salmon sperm DNA, made up to 1 litre with distilled water.

High stringency wash buffer (HSB)

20ml 20 x SSC, 20ml 20% SDS made up to 4 litres with distilled water at 60°C.

DNA FINGERPRINTING:**Labelling buffer**

75µl 1M Tris pH7.5, 35µl 100mM DTT, 35µl 1mM dCTP, 35µl 1mM dATP, 35µl 1mM dTTP, 7µl BSA (10mg/ml), 17.5µl 1M MgCl₂ made up to 1ml with sterile water.

Column buffer

20mM Tris base, 20mM NaCl, 2mM Na₂EDTA, 0.25% SDS. Adjust pH to 7.5 with 1.0M HCl.

Prehybridisation buffer

5g SDS in 500ml 0.5M (pH7.2) Na₂HPO₄

Hybridisation buffer

0.2M Na₂HPO₄ (pH7.2), 1% SDS, 1% BSA (Fraction V), 6% PEG 6000.

Wash solution

0.2M Na₂HPO₄ (pH7.2), 0.1% SDS.

Stripping solution

0.4M NaOH.

Neutralising strip solution

0.2M Tris (pH7.5), 0.1% SDS, 0.1 x SSC.

HPV HYBRIDISATION ANALYSIS:**5 x TBE buffer**

216g Tris base, 110g Boric acid, 80ml 0.5M EDTA (pH8.0) made up to 4 litres with distilled water.

TE buffer

1mM Tris HCl (pH7.5), 1mM EDTA.

Hybridisation solution

5g dextran sulphate dissolved in 25ml doubly distilled water at 65°C (30min), 15ml 20 X SSC, 2.5ml 20% SDS, 800µl 5mg/ml heat denatured salmon sperm DNA.

DNA PLOIDY ANALYSIS:**Citrate buffer**

85.5g sucrose and 11.76g trisodium citrate dissolved in 800ml distilled water, 50ml DMSO, made up to 1 litre with distilled water (pH7.6).

Stock Solution

200mg trisodium citrate, 121mg Tris, 1044mg spermine tetrahydrochloride, 2ml Nonidet P40 dissolved in distilled water to a volume of 2 litres (pH7.6).

Solution A

15mg trypsin in 500ml stock solution (pH7.6)

Stored frozen and brought to room temperature before use.

Solution B

250mg trypsin inhibitor and 50mg ribonuclease A in 500ml stock solution (pH7.6)

Stored frozen and brought to room temperature before use.

Solution C

208mg propidium iodide and 500mg spermine tetrahydrochloride in 500ml stock solution (pH7.6)

Stored frozen and brought to 0°C before use.

APPENDIX 2

PUBLICATIONS

LETTERS TO THE EDITOR

Papillomaviruses, p53, and cervical cancer

SIR,—Dr Crook and colleagues (May 2, p 1070) report that loss of p53 function is important in cervical carcinomas. We have examined 20 primary invasive cervical carcinomas (16 squamous carcinomas, 3 adenocarcinomas, and 1 adenosquamous carcinoma) for human papillomavirus (HPV) status, allele loss on 17p, and p53 mutational status and expression.

Fifteen HPV-positive tumours were identified by PCR with HPV type-specific primers for HPV6, 11, 16, 18, and 33.¹ 5 carcinomas were HPV negative. Allele loss analysis with six restriction fragment-length polymorphic markers on chromosome 17p, revealed losses in only 3 cases—2 were HPV positive and 1 was HPV negative. All 20 cases were informative with one or more of the six probes.

We used a GC-clamp PCR technique for denaturing gradient gel electrophoresis^{2,3} to examine the four hot-spots (A-D) of the p53 gene, where over 80% of p53 mutations have been found in other cancers.⁴ We found no mutations in any of the 20 carcinomas, including the 5 HPV-negative cases and the 3 tumours with 17p allele loss. This finding does not exclude the possibility of mutations outwith regions A-D, but so far few mutations in p53 have been identified in these regions in other cancers. Moreover, in an immunohistochemical technique that uses two human p53-specific monoclonal antibodies, PAb1801 and MAbDO-7,⁵ we were unable to detect accumulated (presumed mutant⁶) p53 protein in any tumour.

The frequent loss of p53 function identified in other malignant diseases^{4,7,8} justifies an investigation of the role of p53 in cervical carcinogenesis. Crook and colleagues' hypothesis⁹ is an attractive one—that in cervical carcinoma, p53 is inactivated either by complexing with HPV E6 protein (in HPV-positive tumours), or by p53 gene mutation in those that are HPV negative. However, our data suggest that a proportion of HPV-negative cervical carcinomas show neither p53 mutation in the conserved regions (A-D) nor allele loss on 17p; these tumours might follow a yet different pathway to the malignant phenotype.

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SIR,—Dr Crook and his colleagues report p53 mutation in 28 primary cervical cancers and an association with human papillomavirus (HPV)-negative tumours. Wildtype p53 can be inactivated by forming complex with the virally encoded HPV16 and 18 oncoprotein E6¹ or by mutation. Three studies on cell lines²⁻⁴ have shown that six different HPV-positive cell lines expressed wildtype p53 whereas two HPV-negative cell lines expressed mutant p53. It is therefore postulated that loss of wildtype p53 function is important in the pathology of cervical cancer and that in the absence of an HPV-encoded gene product, somatic mutation of the gene is required. We have completed a study of 92 tumours from patients with primary cervical carcinomas in which mutations, protein overexpression, and loss of heterozygosity (LOH) of p53 were investigated together with analyses for the presence of HPV16 and 18.⁵ Mutation analysis was done by screening tumour DNA for aberrantly migrating bands with the constant denaturant gel electrophoresis (CDGE) technique,⁶ which detects 100% of the mutations in the screened areas (exons 5-8). Only 2 tumours had mutations. The mutations were confirmed by PCR and direct sequencing with Dynabeads M-280-Streptavidin as solid supports. The presence of HPV16 and 18 was detected by both in-situ hybridisation and PCR of region E6.^{6,7} 1 of the tumours with p53 mutation in codon 173 (GTG→GTA→stop) had no LOH of the p53 locus, was a squamous cell carcinoma stadium Ia, and was positive for HPV18 both on in-situ hybridisation and after PCR. The other tumour with mutation in codon 265 (CTG→TGG→frame shift) was an adenocarcinoma stadium IIIa, was not informative for LOH of the p53 locus, and was positive for HPV16 by PCR only. In the total material, 93% of the samples were HPV16 positive and 22% were HPV18 positive by PCR. Only 4 samples were negative for both HPV16 and 18 and none of these had a p53 mutation within the conserved regions analysed by CDGE. Increased p53 protein was, however, detected in 59% of the tumours by immunostaining with polyclonal antiserum, although most of these tumours (42/47 [89%]) showed overexpression in less than 5% of the cells.

Our data contrast with those of Crook et al and others.²⁻⁴ A much lower frequency of p53 mutations and a somewhat higher frequency of HPV-positive tumours were found in our population.

Cervical carcinoma: low frequency of allele loss at loci implicated in other common malignancies

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Summary Twenty cervical carcinomas were examined for loss of heterozygosity (LOH) using 22 RFLP markers, which mapped to regions of putative oncosuppressor gene loci, identified as candidates in other common solid tumours. Allele losses were identified in six of the eight chromosomal arms examined, but at a significantly lower frequency than that reported in other common solid tumours. No association was observed between allele losses at any chromosomal location and the presence or integration of 'high risk' types of HPV determined by a sensitive, specific PCR method. HPV 16, 18 or 33 were found in the majority (75%) of these tumours. We have looked at only a limited subset of chromosomal regions, but the results, so far, imply that carcinoma of the cervix may arise by different molecular events than other common solid tumours, and support the view that one of the distinctive events may be infection with HPV. Alternatively, similar molecular events may be occurring, but in regions of the genome not yet identified as targets in other solid tumours.

Fundamental changes contributing to carcinogenesis include oncogene activation and tumour suppressor gene inactivation or loss (Knudson, 1989). The location of candidate tumour suppressor genes can be identified by restriction fragment length polymorphism (RFLP) analysis.

A number of putative tumour suppressor genes have now been identified (Ponder, 1988; Sager, 1989), and include the prototype retinoblastoma tumour suppressor gene located at 13q.14; two possible Wilms' tumour genes sited at 11p.13 and 11p.15; the p53 gene at 17p.13; the neurofibromatosis NF-1 gene on 17q; a gene or genes in the region 3p.21 commonly deleted in small cell lung carcinoma and renal carcinoma; the DCC gene deleted in colorectal carcinoma in the region 18q.21-qter; the MCC gene, mutated in colorectal cancer, and the APC gene (responsible for familial adenomatous polyposis coli) both found in the 5q.21 region.

The location of candidate tumour suppressor genes can be identified by the technique of restriction fragment length polymorphism (RFLP) analysis in which labelled polymorphic probes are used as markers to identify individuals heterozygous for specific chromosomal loci. Alleles present in tumour DNA from heterozygous individuals can then be compared with those present in the corresponding constitutional DNA to detect allele loss or loss of heterozygosity (LOH). Such LOH in the vicinity of putative oncosuppressor gene loci has been used as support for Knudson's 'two-hit' hypothesis of carcinogenesis (Knudson, 1989) involving loss of gene function by homozygous inactivation of tumour suppressor genes. Using RFLP analysis, allele losses in the vicinity of oncosuppressor gene loci have now been observed in a high proportion of several common solid human tumours. These losses have been observed in cancers of breast (17p, 17q) (Mackay *et al.*, 1988a; Sato *et al.*, 1991), colon (5q, 17p, 18q) (Vogelstein *et al.*, 1988; Fearon *et al.*, 1990; Ashton-Rickardt *et al.*, 1991; Purdie *et al.*, 1991), ovary (17p, 17q) (Eccles *et al.*, 1990; Russell *et al.*, 1990), lung (3p, 5q) (Naylor *et al.*, 1987; Mori *et al.*, 1989; Ashton-Rickardt *et al.*, 1991), kidney (3p) (Zbar *et al.*, 1987), bladder (9q, 11p, 17p) (Tsai *et al.*, 1990), brain (17p) (Fults *et al.*, 1989), and bone (13q, 17p) (Toguchida *et al.*, 1988; 1989).

In cervical carcinoma, reports of cytogenetic data are few and the numbers of tumours studied have been relatively small. In general, the chromosomal picture has been extremely variable and complex, with complete karyotyping

accomplished in only a few cases. Triploidy and tetraploidy are not uncommon, but no single cytogenetic abnormality has been consistently associated with this tumour type. As in many other types of neoplasia, chromosome 1 has been found to be involved in a non-random fashion, with aberrations comprising both numerical changes and structural rearrangements (Atkin & Baker, 1979; 1982; 1984). These aberrations which include isochromosomes, deletions, duplication, and associated translocations of both the long and short arms, have also been associated with other chromosomes, including chromosomes 3, 4 or 5, 6, 11, 13, 17, 18 and 21 (Atkin & Baker, 1979; 1982; 1984). It has been suggested that chromosome 17 derived markers, frequently present in carcinoma of the cervix, may signify the importance of genes on this chromosome. It has further been postulated that their importance to the development of this cancer may lie in the loss of recessive genes on chromosome 17p (Atkin & Baker, 1989). Furthermore, loss of heterozygosity in primary cervical carcinomas has been reported on chromosomes 3 (Yokota *et al.*, 1989), 11 (Riou *et al.*, 1988; Srivatsan *et al.*, 1991), and 17 (Kaelbling *et al.*, 1992).

In an attempt to learn whether known tumour suppressor genes are involved in the genesis or progression of cervical carcinoma, or whether the presence or integration of human papilloma viruses (HPV) into host DNA influences the pattern of molecular lesions, we examined 20 cases of cervical carcinoma for loss of heterozygosity with 22 RFLP markers. In so doing, we have looked at a limited subset of chromosomal regions on 8 chromosomal arms namely 3p, 5q, 8q, 11p, 13q, 17p, 17q and 18q. With the exception of that on 8q, the markers used all mapped to regions of putative oncosuppressor gene loci, identified in other common solid tumours. The HPV status of each tumour was examined, and an attempt made to relate the presence or integration of HPV to the allele losses observed.

Materials and methods

Twenty paired tumour/blood samples were obtained from consenting patients undergoing Wertheim's hysterectomy or examination under anaesthesia prior to radiotherapy for histologically confirmed and clinically overt cervical carcinomas. The blood samples were used for preparation of constitutional DNA which was used as a matched control for each corresponding tumour.

Three major cervical carcinoma types – squamous carcinoma, adenocarcinoma and adenosquamous carcinoma – of various grades and stages were represented (Table II), and

patients' ages ranged from 23–70 years.

Tumour tissue was snap frozen in liquid nitrogen immediately after surgical removal, and stored at -70°C until DNA extraction. The presence of tumour tissue was confirmed by microscopy. Macroscopic non-cancerous tissue was trimmed from the specimen before DNA extraction, and specimens with less than 70% carcinoma on frozen section were discarded.

Ten μg samples of high molecular weight DNA, extracted from peripheral blood lymphocytes and homogenised fresh tumour tissue, were digested with appropriate restriction endonucleases (Table I), size fractionated by electrophoresis on 0.8% agarose gels, and transferred to Hybond N nylon membranes by Southern blotting.

Polymorphic DNA probes (Table I) were used to compare tumour and constitutional genotypes. Probes were radiolabelled with ^{32}P -dCTP by a standard random multiprimer method (Amersham). Prehybridisation and hybridisation were performed for 2 and 16 h respectively at 65°C using the same buffer ($2.5 \times$ Denhardt's, 0.1% SDS, 0.1% NaPPi, $5 \times$ SSC, 0.01% denatured salmon sperm DNA). Filters were washed at 65°C ($4 \times 15 \text{ min}/0.1 \times$ SSC, 0.1% SDS), autoradiographed at -70°C (Kodak XAR-5 film/Dupont Lightning-Plus Intensifying screens), and the autoradiographs interpreted after 1–14 days.

Primers specific for regions of the E6 gene of HPV types 6, 11, 16, 18 and 33, (Arends *et al.*, 1991) were used in polymerase chain reactions (PCR), with 500 ng samples of tumour DNA as template using 0.5 μl Taq polymerase (Northumbria Biologicals Ltd.) in a volume of 100 μl per reaction. Thirty-two to 35 cycles of annealing ($50/55^{\circ}\text{C}$) (2 min), extension (72°C) (3 min) and denaturation (94°C) (1 min) were preceded by an initial 1.5 min denaturation step and ended with a 10 min extension. The annealing temperature was optimised for, and therefore varied with each HPV type (HPV 6 and 16 – 55°C ; HPV 11, 18 and 33 – 50°C).

PCR products were size fractionated by electrophoresis on 3% agarose gels containing ethidium bromide, and the

presence of the relevant HPV sequences detected by ultraviolet transillumination. A 1 kb lambda marker was electrophoresed concurrently for band size comparison.

Linearised HPV plasmid DNA was radiolabelled and used as a probe in hybridisation experiments with Southern blots of DNA from HPV-positive tumours. Band sizes on the resultant autoradiographs were compared with that of the linearised plasmid HPV DNA, and indicated whether the viral DNA present in the tumour was episomal or integrated.

Results

The results of 211 RFLP analyses of constitutional and tumour DNA samples from 20 patients with cervical carcinoma at 22 polymorphic loci are given in Table I. For each of the 22 marker loci on eight autosomal chromosomal arms, at least two and up to 15 cases were informative. In general, a low overall incidence of LOH was reflected at each oncosuppressor site tested.

Fifteen of the 22 markers revealed LOH in one or more of the informative cases. The frequency of LOH amongst informative cases (Table I) ranged from 7% (pEJ6.6 and PTH) to 30% (Calcitonin). An incidence of 50% LOH occurred with only one probe, pL5.62, in which one of only two informative cases showed LOH. However, over the whole series, of 211 informative loci, only 22 sites (10%), distributed amongst nine tumours, showed LOH.

The majority (11) of the 20 tumours showed no losses at any tested site, and 12 of the examples of LOH were found in just two of the 20 tumours. Clinically and histologically, these two tumours (both squamous carcinomas) did not appear to differ from the others.

Addressing specific chromosomal sites in turn, the combined result with six RFLP markers on chromosome 17p detected LOH at one or more loci in only three of 20 informative cases. All the LOH observed was compatible with deletion involving the p53 gene in the vicinity of 17p.13. No losses were observed amongst 13 informative cases on 17q using the VNTR probe THH59.

No losses were identified amongst 14 informative cases at the locus defined by p68RS2.0 within the Rb gene on chromosome 13q. On chromosome 11p, the calcitonin locus, the c-Ha-ras-1 locus defined by marker pEJ, and those defined by markers PTH and FSH β were analysed. Five losses occurred amongst 19 informative cases. Three cases exhibited LOH at the calcitonin locus; one loss was observed at loci defined by each of the markers pEJ and PTH; and no losses were seen in four cases informative with FSH β .

The combined result of six RFLP markers pL5.62, MC 5.61, pEF 5.44, YN 5.48, MN2.3 and ECB 27 in the 5q.21 region on chromosome 5 revealed LOH in five of 16 informative cases. Losses were detected with pL5.62, pEF5.44, and YN 5.48, while none was observed with MC 5.61, MN 2.3 or ECB 27.

We observed only one LOH amongst four informative cases using the probe pEFD145 which recognises a sequence within the chromosome 3p.21 band.

Two markers were used to detect losses on the long arm of chromosome 18 – pBV15.65 and SAM1.1. Of 12 informative cases, three showed losses – two cases at the SAM 1.1 locus and the other at both loci.

Lastly, one loss was observed in seven informative cases (14%) at the locus defined by marker TL11 on chromosome 8q – so far not implicated as an oncosuppressor site, and hence useful as a 'control' site.

Table II lists the chromosomal arms showing allelic deletion(s) in each tumour, and shows the HPV type(s) that were detected in association with each tumour by PCR analysis. As expected, human papilloma virus was present in three-quarters (15/20), and was integrated rather than episomal in the majority (9/12). Neither viral presence nor its integration correlated with LOH at any specific chromosomal region, nor with the frequency of allele loss seen in any tumour.

Table I Frequency of LOH with 22 RFLP markers in 20 cervical carcinomas

CHR.	Locus	Probe (enzyme)	A/B	Case Nos.
3p	3pter-p21	pEFD 145 (Rsa I)	1/4	6
5q	5q21	pL 5.62 (Bgl II)	1/2	14
5q	5q21-22	MC 5.61 (Msp I)	0/8	–
5q	5q-21	pEF 5.44 (Msp I)	2/11	13 & 16
5q	5q21-22	YN 5.48 (Msp I)	3/13	4, 5 & 16
5q	5q21	MN 2.3 (Msp I)	0/2	–
5q	5q15-21	ECB 27 (Bgl II)	0/6	–
	Any 5q		5/16	
8q	8q22-23	TL 11 (Hind III)	1/7	13
11p	11p15.5	pEJ6.6 (BamH I)	1/15	13
11p	11pter-11p15.4	PTH (Pst I)	1/14	1
11p	11p15.4	Calcitonin (Taq I)	3/10	4, 5 & 16
11p	11p13	FSH β (Hind III)	0/4	–
	Any 11p		5/19	
13q	13q14	*p68RS 2.0 (Rsa I)	0/14	–
17p	17p13.3	*YNZ 22.1 (Taq I)	1/12	13
17p	17p13.3	*YNH 37.3 (Taq I)	1/14	13
17p	17p11.2-cen	pEW 301 (Taq I)	1/10	16
17p	17p12-13	pBHP 53 (BamH I)	1/9	14
17p	17p13	pMCT 35.1 (Msp I)	1/11	16
17p	17p13	C3068 (Hae III)	0/15	–
	Any 17p		3/20	
17q	17q23-25.3	*pTHH 59 (Taq I)	0/13	–
18q	18q21.3	pBV 15.65 (Msp I)	1/5	16
18q	18q21	SAM 1.1 (EcoR I)	3/12	7, 8 & 16
	Any 18q		3/12	

A = No. of cases showing LOH; B = No. of informative cases. *Probe detecting VNTR (Variable No. of Tandem Repeats) sequence.

Table II Allele losses and HPV types in 20 cervical carcinomas studied

Case No.	Histological type	FIGO stage	Location of allele losses	HPV type present
1	A	Ib	11p	–
2	S	Ib	–	16
3	AS	Ib	–	18
4	A	Ib	5q, 11p	16 & 18
5	S	IIIb	5q, 11p	16
6	S	Ib	3p	–
7	S	Ib	18q	16
8	S	Ib	18q	33
9	S	IIIa	–	–
10	A	Ib	–	18
11	S	IVa	–	16
12	S	Ib	–	16
13	S	Ib	5q, 8q, 11p, 17p	16 & 18
14	S	Ib	5q, 17p	–
15	S	Ib	–	–
16	S	Ib	5q, 11p, 17p, 18q	16
17	S	Ib	–	16
18	S	Ib	–	18
19	S	Ib	–	16
20	S	IIIb	–	16

S = Squamous; AS = Adenosquamous; A = Adenocarcinoma.

Discussion

The unimpressive incidence of LOH at the known oncosuppressor sites in this series of cervical carcinomas contrasts with that reported in other major solid tumours (Table III). Although only a limited subset of chromosomal regions was examined, the results suggest that these oncosuppressor genes, commonly implicated in other human tumours, do not play a significant role in cervical carcinogenesis.

Allele deletions on chromosome 17p have been reported in up to 61% of breast carcinomas (Mackay *et al.*, 1988a); 73.1% of osteosarcomas (Toguchida *et al.*, 1989); 75% of colonic carcinomas (Vogelstein *et al.*, 1988); 50–60% of epithelial ovarian carcinomas (Eccles *et al.*, 1990; Russell *et al.*, 1990); up to 55% of brain tumours (Fults *et al.*, 1989) and 63% of bladder carcinomas (Tsai *et al.*, 1990) – suggesting the presence of a tumour suppressor gene that is involved in a carcinogenetic mechanism common to all of these tumours. Our data show only 15% of informative tumours with 17p allele loss; a similar proportion to that

identified in a recently published series of cervical carcinomas (Kaelbling *et al.*, 1992), but a significantly lower proportion ($P < 0.05$; Fisher's Exact Test) than that observed using similar probes in tumours of breast, bladder, ovary, bone, and colon. This may imply that any association between 17p allele loss and carcinogenesis does not extend to cervical carcinoma.

An association between the HPV E6 and E7 genes and the p53 (17p) and Rb (13q) genes respectively, has been suggested (Banks *et al.*, 1990; Crook *et al.*, 1991; Scheffner *et al.*, 1991). Inactivated p53 protein has been associated with HPV 16 E6 oncoprotein complex formation (Scheffner *et al.*, 1991) while the Rb gene cellular protein has been associated with HPV 16 E7 protein (Banks *et al.*, 1990; Scheffner *et al.*, 1991). In studies on cervical carcinoma cell lines, and more recently on cervical tumour tissue, wild type p53 mRNA and DNA were sequenced from HPV positive cell lines and tumours respectively, while the mutated form was detected only in those that were HPV negative; suggesting that alternative and mutually exclusive routes for altering p53 function are adopted in cervical carcinogenesis: mutation, or complex formation with HPV E6 (Crook *et al.*, 1991; 1992). The results of our study are not wholly consistent with this proposition, in that two of three cases showing LOH at 17p, in the vicinity of the p53 gene, were HPV positive. However, it is possible that p53 may yet exert a dominant negative effect, with its function altered by mutation in the absence of LOH on chromosome 17p.

Research on cervical carcinoma cell lines has suggested a role for genes on chromosome 11. Microcell transfer of a single copy of fibroblast chromosome 11 into tumorigenic HeLa cells converted them into a non-tumorigenic state (Saxon *et al.*, 1986); and a putative tumour suppressor gene identified in HeLa cells has been mapped to the chromosome 11q.13 region (Srivatsan *et al.*, 1991). Loss of heterozygosity on chromosome 11, in 30% of cervical carcinoma cases, has been reported in a recent study (Srivatsan *et al.*, 1991); while 36% LOH on chromosome 11p had been reported in a previous series (Riou *et al.*, 1988). Our analysis using four markers on the short arm of chromosome 11 revealed a frequency of LOH lower than that observed with equivalent probes in breast cancer; and, for three of the four probes used, the incidence of LOH was distinctly lower than that observed at the 'innocent' locus on 8q. It therefore appears unlikely that an oncosuppressor gene of major importance to cervical carcinogenesis resides on the short arm of chromosome 11. An analysis of the long arm of this chromosome

Table III Comparative loss of heterozygosity: Published series of other tumour types vs this series of cervical carcinomas

CHR	Tumour type (Ref.)	Published series – other tumours		This series – cervical carcinoma		
		A/B	%	A/B ^a	%	P
17p	Breast (Devilee <i>et al.</i> , 1989)	30/49	61	1/12	8	0.001
	Bladder (Tsai <i>et al.</i> , 1990)	15/24	63	1/12	8	0.004
	Ovary (Eccles <i>et al.</i> , 1992)	10/18	56	1/9	11	0.042
	Bone (Toguchida <i>et al.</i> , 1989)	19/26	73	3/20	15	<0.001
	Colon (Vogelstein <i>et al.</i> , 1988)	45/60	75	3/20	15	<0.001
17q	Ovary (Eccles <i>et al.</i> , 1990)	10/13	77	0/13	0	<0.001
13q	Breast (Devilee <i>et al.</i> , 1989)	12/32	38	0/14	0	<0.001
	Bone (Toguchida <i>et al.</i> , 1988)	13/30	43	0/14	0	<0.001
11p	Breast (Mackay <i>et al.</i> , 1988b)	4/7	57	1/14	7	0.025
	Bladder (Tsai <i>et al.</i> , 1990)	9/23	39	1/15	7	NS
5q	Colon (Ashton-Rickardt <i>et al.</i> , 1991)	44/106	42	5/16	31	NS
3p	Lung (Naylor <i>et al.</i> , 1987)	9/9	100	1/4	25	0.014
	Kidney (Zbar <i>et al.</i> , 1987)	11/11	100	1/4	25	0.009
18q	Colon (Fearon <i>et al.</i> , 1990)	29/41	71	1/5	20	0.018

CHR = Chromosome. A = No. of cases with loss of heterozygosity. B = No. of informative cases. % = Percentage of informative cases with loss of heterozygosity. ^aSame probe/s used in this series as in published series compared. P = P-value (Fisher's Exact Test). NS = Not statistically significant.

was not performed because of the unavailability of DNA probes.

Similarly, losses on chromosome 5q in the region of the APC and MCC tumour suppressor genes, and on 18q in the vicinity of the DCC gene have been associated with colorectal cancer at levels of 41.5% (Ashton-Rickardt *et al.*, 1991), and 71.0% (Fearon *et al.*, 1990) respectively, but, a high level of LOH was not detected at these loci in cervical carcinoma.

Previously published work in this field reported the occurrence of allele losses in nine of nine informative cases on chromosome 3 at p14-21 (Yokota *et al.*, 1989). Although our results do not directly refute this finding, as the probe used by Yokota *et al.*, was not available to us, we demonstrated no significant LOH at a locus just bordering this region (3p.21).

Human papilloma viruses, especially types 16 and 18, have long been associated with malignancy in the cervix (Bosch *et al.*, 1989; zur Hausen, 1989; Chang, 1990; Singer & Jenkins, 1991). This association has been confirmed by PCR in 75% of the cases in the present study. However, our finding that common HPV types 6, 11, 16, 18 and 33 were absent in 25% of the tumours examined suggests that HPV infection may

not be a prerequisite for malignant transformation in the cervix. Both HPV-positive and HPV-negative tumours showed a low frequency of LOH, and there was no consistent relationship between LOH and the integrated or episomal state of the virus. This suggests that cervical carcinoma cells arrive at the malignant phenotype by pathways which are probably different from those identified in other common solid tumours, but which do not necessarily involve the common HPV types.

If oncosuppressor genes are involved in cervical carcinogenesis, they are probably found at loci different from those commonly deleted in other solid tumours. Alternatively, a different mode of carcinogenesis, perhaps involving HPV or other virus types, may be involved.

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